ROLE OF CYCLIN E IN THE PATHOGENESIS OF HEPATOCELLULAR CARCINOMA

Sharon Pok

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Statement of originality

The candidate claims originality over the results and analyses presented in this thesis, except for work that has been contributed by collaborators, where contribution is duly acknowledged. This work has been conducted in the Liver Laboratory within The Australian National University Medical School located at The Canberra Hospital from February 2008 to March 2012 under the supervision of Professor Narcissus Teoh and Professor Geoffrey Farrell. The work is original and has not been previously used to obtain a degree.

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Abstract

Hepatocellular carcinoma (HCC), or primary liver cancer, is the fifth most common cancer worldwide and the third most common cause of cancer mortality (El-Serag 2012). The development of HCC is thought to be a multi-staged process that involves several risk factors including the chronic hepatitis B and C infection, carcinogen exposure, metabolic disease, excessive alcohol consumption and male gender. Accumulation of genetic and epigenetic alterations with DNA-damaged hepatocytes can also contribute to the molecular pathogenesis of HCC. A better understanding of molecular mechanisms associated with HCC could ultimately improve our current strategies for screening and targeted therapy of this disease.

Array comparative genomic hybridisation studies in murine diethylnitrosamine (DEN)-induced HCC have identified *ccne1* (cyclin E) as a candidate gene associated in accelerated liver carcinogenesis (Teoh *et al.* 2008). In order to investigate the role of cyclin E and its impact on hepatocyte cell cycle regulation in early HCC development, we employed a well-known and highly reproducible rodent model of DEN-induced hepatocarcinogenesis. C57BL/6J male mice were injected with DEN (10 mg/kg i.p.), age day 12 – 15. In this model, male animals develop hepatocyte dysplasia at 6 mths and HCC in >90%. Transcript and protein expression of cyclin E was evident as early as 6 mths in dysplastic nodules (DNs), and significantly increased in HCCs. In contrast, there was little or undetectable cyclin E in normal liver and liver surrounding HCCs at all timepoints. Glutathione S transferase-pi form and cyclin E expression co-localised in DNs from liver in mice at 6 and 9 mths. Cyclin E/cdk2 kinase activity was also significantly upregulated in DNs, while increased proliferative activity by cyclin D1 and proliferating cell nuclear antigen (PCNA) in HCCs was observed at 9 mths, a timepoint where there was maximal p53 and p21 tumour suppressor expression. Aberrant cyclin E

protein expression, including low molecular weight (LMW) isoforms were detected in HCCs and liver surrounding HCCs. Interestingly, sequencing analyses of *p53* revealed a 1093 – 1361 nucleotide deletion in up to 90% of DNs, causing dysfunctional p53 nuclear localisation and export signalling. Because p53 directly signals to p21, co-immunoprecipitation studies were performed and revealed preferential binding of p21 to cyclin D1, rather than cyclin E, thereby allowing "escape" from the G1/S checkpoint.

To directly test whether cyclin E regulates p53 expression in HCCs derived from DEN-treated male mice at 9 mths, we conducted cyclin E knockdown in primary HCC cells. This strategy resulted in increased p53 and p21 expression, as well as significant diminution of Bcl-xL, the p53-induced anti-apoptotic marker. Cell viability tetrazolium/formazan assay was significantly impaired in cyclin E RNAi targeted primary HCC cells. Conversely, chemical inhibition of p53 by pfithrin-α, augmented cyclin E, PCNA and Bcl-xL protein expression whilst cell viability was restored following co-treatment with MG-262, a 26S proteasome inhibitor. In contrast, overexpressing cyclin E in naïve primary hepatocytes enhanced PCNA expression, increased hepatocyte viability, downregulated p53 and its downstream signalling intermediate, p21.

We next determined whether miRNA-34, a co-regulator of cyclin E and p53, was instrumental in the reciprocity between cyclin E and p53 as key "drivers" of hepatocarcinogenesis. Dysplastic liver and HCCs obtained from DEN-injected male mice were assayed for miR-34a,b,c. miR-34a and c were significantly upregulated in HCCs and dysplastic liver compared with normal liver. Similar trends were noted for miR-34a,b,c in human hepatitis C-related HCCs when compared with normal human liver. Importantly, this was associated with significantly enhanced *cyclin E* and *p53* mRNA expression in human HCCs compared to normal and cirrhotic liver.

In this murine model, there was disproportional and upregulation of functionally active cyclin E, miR-34a,c in DNs and early HCCs with congruent loss of p53 function associated with cell cycle checkpoint failure, diminished apoptosis and increased proliferative drive. In human HCV-related HCCs, miR-34a, *p53* and *cyclin E* transcript levels were universally upregulated. When we performed *in vitro* experiments in murine primary hepatocytes and primary HCC cells, knocking down or overexpressing cyclin E did not affect miR-34 expression. However, stabilising p53 with MG-262 enhanced miR-34a,c expression (though not significant), whilst inhibiting p53 using pfithrin-α significantly reduced miR-34a,c. miR-34 may provide a plausible link to increased cyclin E expression, activity and increased proliferative drive in dysplastic and neoplastic liver in mice and humans.

Gender disparity in human HCC is well described, with a strong male predominance. However, the role of sex hormones in hepatocarcinogenesis remains poorly defined. In order to determine if there are gender differences in the expression of cyclin E, effects on cell cycle regulators and tumour suppressors, dysplastic liver and HCCs were studied in DEN-treated C57BL/6J female mice. These mice displayed a significant reduction in dysplastic hepatocytes compared with intact DEN-treated males at 3, 6 mths, while HCC incidence, number and size of tumours were significantly diminished in females at up to 15 mths. In carcinogen-treated female mice, cyclin E (native and LMW isoforms) protein expression and kinase activity were reduced compared to males at 6 – 12 mths, with concomitant reduction in hepatocyte proliferation by PCNA and cyclin D1 expression. Unlike male mice, G1/S checkpoint is evident by robust p53-mediated apoptosis.

To ascertain if these differences are attributable to the effects of oestradiol/progesterone (E/P) and/or testosterone, we conducted hormonal manipulation

studies using the same carcinogen-model by performing ovariectomy in female animals and orchidectomy in male mice, in which some animals received E/P or testosterone supplementation. Castration of DEN-injected male mice resulted in a loss of cyclin E LMW isoforms compared to intact males, diminution of cyclin E kinase activity and phospho-retinoblastoma expression. There was also induction of p53-mediated apoptosis in dysplastic hepatocytes, leading to a reduction in number of DNs by 6 mths. These anti-proliferative and pro-apoptotic effects were magnified by E/P replacement in castrated DEN-treated males. In contrast, testosterone-replacement in ovariectomised-female mice exhibited accelerated hepatocarcinogenesis compared to intact female DEN-treated animals and displayed LMW cyclin E isoforms similar to those detected in DEN-injected intact males.

In further analyses, there was increased oestrogen receptor-α (*ERα*) transcript and protein expression in HCCs derived from DEN-injected intact male mice, and in dysplastic liver from castrated male mice replaced with E/P. E/P replacement and testosterone withdrawal were associated with ERα expression, the loss of cyclin E LMW isoforms, intact cdk2 expression, functional G1/S checkpoint control and induction of p53-mediated apoptotic cell death in preneoplastic hepatocytes. Further, oestrogen (E2) stimulation had varying effects on cell cycle regulation and viability in primary hepatocytes and HCC cells. As there was little to no significant correlation between ERα and its downstream target, c-*myc* transcript levels, we propose that E2/ERα signalling may be operative via other pathways to subsequently activate p53. These findings open up tantalising avenues to further explore the inter-regulatory signalling pathways between E2/ERα, cell cycle regulators and the tumour suppressor, p53.

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Publications, presentations and awards

Manuscript accepted for publication

S Pok, V Wen, NA Shackel, A Alsop, P Pyakurel, A Fahrer, GC Farrell, NC Teoh. (2013) *Cyclin E facilitates dysplastic hepatocytes to bypass G1/S checkpoint in hepatocarcinogenesis*. J Gastro Hepatol; in press.

Published Abstracts

S Pok, P Pyakurel, L Maddocks, NC Teoh. (2009). *Cyclin E in precursor lesions in hepatocellular carcinoma*. J Gastro Hepatol 24 (S2): A278-A279.

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S Pok, L Bala, AM Fahrer, GC Farrell, NC Teoh. (2010). *Crucial interactions between Cyclin E and p53 tumour suppressor gene in hepatocarcinogenesis*. J Gastro Hepatol 25 (S3): A5.

S Pok, HJ Wong, VW Wen, NA Shackel, GC Farrell, NC Teoh. (2011). *Cyclin E and p53 interactions associated with upregulation of miR-34 facilitate proliferative drive and suppress apoptosis in liver carcinogenesis.* J Gastro Hepatol 26 (S4): 3.

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Conference Presentations

S Pok[#], P Pyakurel, NC Teoh. (2009). *Role of Cyclin E in hepatocellular carcinoma*. Australian Liver Association Hepatology and Luminal Workshop, May 1 – 3, Yarra Valley, Australia. Oral presentation.

S Pok, P Pyakurel, L Maddocks, NC Teoh. (2009). *Role of Cyclin E in hepatocarcinogenesis: A study done using a carcinogen-induced murine model*. Australian Scientific Medical Research Young Investigator Forum, Jun 2, Canberra, Australia. Poster.

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S Pok, P Pyakurel, L Bala, NC Teoh. (2010). *Cyclin E & p53 interactions in liver carcinogenesis*. Australian Scientific Medical Research Young Investigator Forum, Jun 1, Canberra, Australia. Oral presentation.

S Pok, GC Farrell, NC Teoh. (2010). *Crucial interactions between Cyclin E and p53 tumour suppressor gene in hepatocarcinogenesis*. Asia Pacific Digestive Week, Sept 19 – 21, Kuala Lumpur, Malaysia. Poster.

S Pok, L Bala, A Fahrer, GC Farrell, NC Teoh. (2010). *Crucial interactions between Cyclin E and p53 tumour suppressor gene in hepatocarcinogenesis*. Australian Gastroenterology Week, Oct 20 – 23, Gold Coast, Australia. Oral presentation

S Pok, HJ Wong, VW Wen, NA Shackel, GC Farrell, NC Teoh. (2011). *Cyclin E and p53 interactions associated with upregulation of miR-34 facilitate proliferative drive and suppress apoptosis in liver carcinogenesis*. Australian Gastroenterology Week, Sept 12 – 15, Brisbane, Australia. Oral presentation.

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S Pok, HJ Wong, VW Wen, NA Shackel, GC Farrell, NC Teoh. (2011). *Crucial interactions between cyclin E and p53 tumour suppressor in facilitating proliferative drive and suppressing apoptosis in murine hepatocarcinogenesis*. Asia Pacific Digestive Week, Oct 1 – 4, Singapore. Poster.

S Pok, VA Barn, GC Farrell, NC Teoh. (2012). *Gender differences in hepatocarcinogenesis: effect of oestradiol, progesterone and testosterone on hepatocyte cell cycle regulators*. Canberra Health Annual Research Meeting, Aug 14 – 17, Canberra, Australia. Paper of Merit. Oral presentation.

S Pok, VA Barn, GC Farrell, NC Teoh. (2012). *Oestradiol and progesterone attenuate diethylnitrosamine-induced liver carcinogenesis in association with restitution of G1/S checkpoint control.* Australian Gastroenterology Week, Oct 11 – 14, Adelaide, Australia. Oral presentation.

[#] Presenting author is underlined.

Awards

- **Best Poster Presentation** at the Australian Scientific Medical Research (ASMR) Medical Research Week Australian Capital Territory (ACT) region. June 2009.
- **Fieldwork funding** by ANU Medical School at Cancer Research Program at the Garvan Institute of Medical Research (Sydney, NSW, Australia). Sept 2009.
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- **Runner-up award for Oral Presentation** at the ASMR Medical Research Week ACT region. June 2011.
- **Winner of Poster of Merit** at Australian Gastroenterology Week, Brisbane, QLD, Australia. Sept 2011.

Abbreviations

4-OH-Tam	4-hydroxytamoxifen
Acox	Acyl-CoA oxidase
ADP	Adenosine diphosphate
AF1	Activation function 1
AF2	Activation function 2
AFB_1	Aflatoxin B1
AFP	α-fetoprotein
AH	Adenomatous hyperplasia
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APC	Adenomatous polyposi coli
AR	Androgen receptor
ARE	Androgen response element
ARF	Alternative reading frame
AST	Aspartate aminotransferase
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related
B/B_0	Bound/maximum bound
BAC	Bacterial artificial chromosome
BH	Bcl2 homology
BMI	Body mass index
bp	Base-pairs
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAK	Cdk-activating kinase
CCRK	Cell cycle-related kinase
cdc25A	Cell cycle protein 25A
Cdk	Cyclin-dependent kinase
cDNA	Complimentary deoxyribonucleic acid
CGH	Comparative genomic hybridisation
CHB	Chronic hepatitis B
CIN	Chromosomal instability
CK-18	Cytokeratin-18
CKI	Cdk inhibitor
COMT	Catechol O-methyltransferase
CYP	Cytochrome P450
DAB	Diaminobenzidine

DAPI	4,6-diamidino-2-phenylindole
DEN	Diethylnitrosamine
DEP.C	Diethylpyrocarbonate
DES	Diethylstilbestrol
DGCP	Des-γ-carboxyprothrombin
DHEA	Dehydroepiandrosterone
DISC	Death-inducing-signalling-complex
DMSO	Dimethyl sulfoxide
DN	Dysplastic nodule
DNA	Deoxyribonucleic acid
DNA-PK_{CS}	DNA protein kinase (catalytic subunit)
DSB	Double strand break
DTT	Dithiothreitol
E/P	Oestradiol/progesterone
E2	Oestradiol
EDTA	Ethylenediaminetetraacetic acid
EE	17-α ethinyl oestradiol
EGF	Epidermal growth factor
EGR1	Early growth response protein 1
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor
ERE	Oestrogen response element
ERK	Extracellular signal-regulated kinase
ERK2	Extracellular signal-regulated kinase-2
ERα	Oestrogen receptor-α
EtOH	Ethanol
FADD	Fas associated protein with death domain
FBS	Fetal bovine serum
Flt3	FMS-like tyrosine kinase 3
FNH	Focal nodular hyperplasia
g	G force, relative centrifugal force
G	Gauge
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gpc3	Glypican-3
Grb2	Growth factor receptor-bound protein 2
GSH	Glutathione
GSK-3β	Glycogen synthase kinase-3β
GST-pi	Glutathione-S-transferase pi form
GTP	Guanosine triphosphate

GWAS	Genome-wide association study
H&E	Haemotoxylin & eosin
HA	Hepatic adenoma
HAUSP	Herpes-virus-associated ubiquitin-specific protease
HBeAg	Hepatitis B e-antigen
HBSS	Hank's balanced salt solution
HBV	Hepatitis B virus
HBVCP	HBV core protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL	High density lipoprotein
hdm2	Human double minute homolog 2
HGDN	High grade dysplastic nodules
HKMT	Histone lysine methyltransferase
HPV	Hepatic portal vein
HR	Homologous recombination
HRP	Horseradish peroxidase
i.p.	Intra-peritoneally
IARC	International agency for research on cancer
IB	Immunoblotting
IGF1	Insulin-like growth factor-1
IGF2R	Insulin-like growth factor 2 receptor
IHC	Immunohistochemistry
IP3	Inositol triphosphate
IVC	Inferior vena cava
IWP	International working party
LB	Luria bertani
LBWR	Liver-to-body weight ratio
LCM	Laser capture microdissection
LDL	Low density lipoprotein
LGDN	Low grade dysplastic nodule
LMW	Lower molecular weight
LOH	Loss of heterozygosity
lyve1	Lymphatic vessel endothelial hyaluronan 1
MAP	MEK activating protein
max	Myc-associated factor X
MCS	Multiple cloning site
mdm2	Mouse double minute 2
mdr2	Multiple drug resistance 2
MEF	Mouse embryonic fibroblast

MEK	Methyl ethyl ketone
miRNA	Micro ribonucleic acid
MRN	Mre11/Rad50/Nbs1
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NHEJ	Non-homologous end joining
NPM	Nucleophosmin
NS	Not significant
OMM	Outer mitrochondrial membrane
ORCX	Orchidectomy
OVX	Ovariectomy
p53i	p53 inactivation
PARP1	Poly (ADP-ribose) polymerase 1
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGFRβ	Platelet-derived growth factor receptor-β
PFT	Pfithrin-α
Pg	Progesterone
PgR	Progesterone receptor
PH	Partial hepatectomy
PI3K	Phosphatidylinositol 3-kinase
PIKK	PI3K-like protein kinase
PKCα	Protein kinase C-α
PPARα	Peroxisome proliferator-activated receptor-α
PPARγ	Peroxisome proliferator-activated receptor-γ
pRb	Phosphorylated retinoblastoma
PRE	Pg response element
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene fluoride
RasGEF	Ras guanine nucleotide exchange factor
Rb	Retinoblastoma
RE	Response element
RFLP	Restriction fragment length polymorphism
RISC	Ribonucleic acid-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species

RPA	Replication protein A
rpm	Rotations per minute
RT-PCR	Reverse transcription-polymerase chain reaction
s.c.	Subcutaneously
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH2	Src homology 2
SIRT1	Silent information regulator 1
SNP	Single nucleotide polymorphism
SOS	Son of sevenless
ssDNA	Single-stranded DNA
Stat3	Signal transducer and activator of transcription 3
SUMO	Small ubiquitin-related modifier
T	Testosterone
T2DM	Type 2 diabetes mellitus
TAA	Thioacetamide
TBST	Tris-based saline with Tween-20
TCF	T-cell factor
TEMED	Tetramethylethylenediamine
Tfm	Testicular feminisation
TGFα	Transforming growth factor-α
TGFβ	Transforming growth factor-β
TIN2	TRF1-interacting nuclear protein 2
TNFR	TNF receptor
TNFα	Tumour necrosis factor-α
TPX2	Targeting protein for Xklp2
TRAIL	TNF-related apoptosis-inducing ligand
TRF1	Telomeric repeat-binding factor 1
TRF2	Telomeric repeat-binding factor 2
UNG	Uracil N-glycosylase
VEGFR	Vascular endothelial growth factor receptor
vER	Variant oestrogen receptor
WHO	World Health Organisation
WT	Wildtype

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