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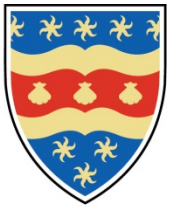

UNIVERSITY OF
PLYMOUTH

AN ASSESSMENT OF UNIVERSAL TUMOUR ASSOCIATED ANTIGENS IN PRIMARY LIVER NEOPLASMS

by

OLIVER DOUGAL GILLIATT RUPAR

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This work is dedicated to Lynn, Beatrix and Dobby.

AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

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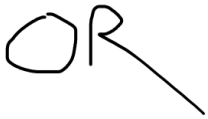

Date 28th March 2021

Oliver Dougal Gilliatt Rupar

An assessment of universal tumour associated antigens in primary liver neoplasms.

Abstract

Rates of primary liver cancer are increasing in the Western world, a fact yet to be reflected in research with most currently undertaken in Asia. Whilst here in the UK, during a diagnostic and therapeutic workup within the NHS, surgically resected tumours are routinely kept in storage for up to thirty years. Meaning there is a huge, currently unused, resource that could potentially be included in clinical research.

The regional specialist centre in the Southwest of England has been performing liver resections since 2005, no basic-science research has been performed using these archived specimens. In this research I have assessed the presence of genetic alterations, attempted to quantify the transcriptome, and measured protein expression in patients with liver cancer using a digital pathology platform. Telomerase and Survivin, the two targets of these endeavours, have previously been shown to be expressed in numerous cancer types, earning the title ‘universal tumour associated antigen.’ The techniques used in this research project are, mostly, already used in healthcare diagnostics, meaning there is potential for vastly increasing the power of these results should the study be increased.

Genetic alterations in the promoter sequences were amplified and sequenced using DNA from archived samples. Attempts to quantify the transcribed component which have been locked away in tiny exosomes (which lack degradation enzymes) were made using a quantitative polymerase chain reaction. Protein expression was detected in tumours and background liver tissues using immunohistochemistry and quantified using digital pathology techniques on whole-slide images. Haematological protein levels were assessed using the enzyme linked immunosorbent assay. All of these characteristics

were then compared with clinical measures such as tumour size, grade, stage, vascular invasion, overall survival, and diseases associated with liver carcinogenesis.

Lessons learned from my work, particularly the techniques used on the source material, could be used in any NHS department without the need for significant financial investment required for a formal research facility. Access to these precious resources allows a more accurate representation of these antigens in the local clinical cohort.

Below I have provided evidence that Telomerase promoter mutations are an HCC-specific alteration, and are present in tumours with vascular invasion. There is also early evidence that these mutations may correlate with a reduced overall survival. The Survivin promoter has been found to be a germline characteristic, whilst Survivin protein expression has been found to correlate with numerous adverse clinical features including tumour stage, grade, vascular invasion, perineural invasion and overall survival. These results are very encouraging and could possibly even be used as a risk-stratification tool during future routine clinical liver tumour workup, as an aid to identify patients at a higher risk of adverse clinical outcomes.

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Abbreviations

*	Pearson's Chi Square
AFP	Alpha-foetoprotein
AJCC	American Joint Committee on Cancer
Akt	Protein kinase B
ALT	Alternative lengthening of Telomeres
ARID1A	AT-rich interaction domain 1A gene
ASR	Age standardised rates
BAP1	BRCA1-associated protein 1
BCLC	Barcelona Clinic Liver Cancer (stage)
BIBR1532	Small molecule Telomerase inhibitor
BIRC5	Gene encoding Survivin
BG	Background (liver)
BIR	Baculovirus inhibitor of apoptosis repeat
BLAST	Basic local alignment search tool
BMI	Body mass index
BP	Base pair
BRAF	B-Raf proto-oncogene
C228T	Human Telomerase promoter mutation at nucleotide 1295228, 124 bases upstream of the transcription start site of the TERT gene
C250T	Human Telomerase promoter mutation at nucleotide 1295250, 146 bases upstream of the transcription start site of the TERT gene
CA19-9	Carbohydrate antigen 19-9
CAFs	Cancer associated fibroblasts
CCA	Cholangiocellular carcinoma
CEA	Carcinoembryonic antigen
CD9	One of four tetraspanins, an exosomal marker
CD63	One of four tetraspanins, an exosomal marker
CD81	One of four tetraspanins, an exosomal marker
CD82	One of four tetraspanins, an exosomal marker
CDE/CHR	Cell-cycle-dependent element/ cell-cycle gene homology region
cDNA	Complementary DNA

CI	Confidence interval
CPC	Chromosomal passenger complex
CRC	Colorectal carcinoma
Crm1	Chromosome region maintenance 1
CTNNB1	Gene encoding Beta-catenin 1
DAA	Direct acting antivirals
DAB	3,3′-Diaminobenzidine
dCCA	Distal Cholangiocellular carcinoma
ddPCR	Droplet-digital polymerase chain reaction
DEB-TACE	Drug eluting bead transarterial chemoembolisation
DNA	Deoxyribose Nucleic Acid
eCCA	Extrahepatic CCA
ECM	Extracellular matrix EDTA
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase
ES	Edmondson-Steiner (grade)
ETS	E-Twenty-Six
F	Forward (sequence)
FFPE	Formalin fixed paraffin embedded
FGFR	Fibroblast growth factor receptor
FL-HCC	Fibrolamellar Hepatocellular carcinoma
GABPA	GA binding protein transcription factor subunit alpha
GAR1	GAR1 ribonucleoprotein
G2/M	Second growth phase/ Mitosis (in the cell cycle)
GPC-3	Glypican-3
GRC37/38	Genome Reference Consortium Human genome build 37/38
HBV	Hepatitis B Virus
HBXIP	Hepatitis B virus X-interacting protein
HCA	Hepatocellular adenoma
HCC	Hepatocellular carcinoma

HCC-CCA	Mixed hepatocellular/cholangiocellular carcinoma
HCV	Hepatitis C Virus
HDAC	Histone deacetylases
HPA	Human protein atlas
HPRT1	Hypoxanthine phosphoribosyltransferase 1, a housekeeping gene
H-Score	The histological score (0 – 300)
hTERT	Human Telomerase reverse transcriptase
Huh7.5	Human hepatoma-derived cell line 7.5
HV	Healthy volunteer
IAP	Inhibitor of apoptosis
iCCA	Intrahepatic Cholangiocellular carcinoma
IDH-1/2	Isocitrate dehydrogenase 1/2
IHC	Immunohistochemistry
INCEPT	Inner centromere protein
KAPA 2G	A synthetic DNA Polymerase
KASP	Kompetitive allele-specific polymerase chain reaction
KEAP1	Kelch-like ECH associated protein 1
KM	Kaplan-Meier
KML001	An arsenic-based compound that inhibits Telomerase
KRAS	KRAS proto-oncogene
LIMS	Laboratory information management system
LN	Lymph node
LC/MS	Liquid chromatography mass spectrometry
MAPK	Mitogen-activated protein kinase
MGMT	O(6)-methylguanine-DNA methyltransferase
microRNA	A single-stranded non-coding RNA, containing about 22 nucleotides
MLL3	Mixed-lineage leukaemia protein 3
MMD	Mild/Moderately differentiated
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
MVs	Microvesicles
MVE	Multi-vesicular endosome
MWA	Microwave ablation

NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NES	Nuclear export signal
NHP2	H/ACA ribonucleoprotein complex subunit 2
NOP10	Nucleolar protein 10
NOS	Not otherwise specified
NRF2	Nuclear factor erythroid 2-related factor 2
NS	Not stated
OD	Optical density
PBC	Primary biliary cirrhosis
PBRM1	Polybromo1
pCCA	Perihilar Cholangiocellular carcinoma
PCR	Polymerase chain reaction
PEG3	Paternally expressed gene 3
PD	Poorly differentiated
PHH	Primary human hepatocytes
Phred Score	An integer value representing the estimated probability of error in a base-call. Also known as the Q score.
PLC	Primary liver cancer
PLN	Primary liver neoplasm
POT1	Human protection of telomeres 1
PRKACA	Protein kinase cAMP-activated catalytic subunit alpha
PSC	Primary sclerosing cholangitis
pSurv	Survivin promoter
$pTERT^{Mut/WT}$	Telomerase promoter Mutant/ Wild type
R	Reverse (sequence)
R0	Fully resected tumour
R1	Tumour cells present at resection margin
RAF kinases	Rapidly accelerated fibrosarcoma kinases
RAP	Repeat addition processivity
RAP1	Ras-related protein 1
RAS	Family of RAS genes: HRAS, NRAS & KRAS
RD&I	Research, development and innovation
REC	Research ethics committee

RFA	Radiofrequency ablation
RFLP	Restriction fragment length polymorphism
RNF43	Ring finger protein 43
ROBO2	Roundabout guidance receptor 2
ROI	Region of interest
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
scaRNP	Small Cajal body ribonucleoprotein
S.D.	Standard deviation
SMAD4	Mothers against decapentaplegic homolog 4
SNP	Single nucleotide polymorphism
smac/DIABLO	Second Mitochondria-derived Activator of Caspases/ Direct IAP-Binding protein with Low PI
SPSS	The statistical software package: statistical package for the social sciences
ssDNA	Single stranded DNA
$Surv^{High/Low}$	High/Low Survivin expression
SVR	Sustained viral response
T	Tumour
T_a	Annealing temperature
TAA	Tumour associated antigen
TACE	Transarterial chemoembolization
TARE	Transarterial radioembolization
TBS-T	Tris buffered saline-Tween-20
TERT	Telomerase reverse transcriptase – a catalytic enzyme
$TERT^{High/Low}$	High/Low Telomerase protein expression
TIN2	TERF-interacting nuclear factor 2
T_m	Melting temperature
TME	Tumour microenvironment
TNF-R	Tumour necrosis factor-receptor
TNM	Tumour, Nodes, Metastasis
TP53	Tumour protein 53
TPP1	Tripeptidyl peptidase1
TR/ TERC	Telomerase-RNA

TRAP	Telomerase repeat amplification protocol
TRF1/2	Telomeric repeat factor 1/2
TRIS base	2-Amino-2-(hydroxymethyl)-1,3-propanediol
UHP	University Hospitals Plymouth
UTAA	Universal tumour associated antigen
VI	Vascular invasion
WD	Well differentiated
WHO	World Health Organisation
Wnt	Wingless-related integration site
WT	Wildtype
XIAP	X-linked inhibitor of apoptosis

1 Introduction

Primary liver cancers (PLCs) are the sixth most common cancer type and, globally, the second most common cause of cancer related deaths (Bray et al., 2018; Pezzuto et al., 2017). The two most common sub-types of primary liver cancer are Hepatocellular Carcinoma (HCC) and Cholangiocellular Carcinoma (CCA) which account for the vast majority (more than 99%) of PLCs. HCCs are tumours of the liver cells (hepatocytes) and comprise around 80-90% of all PLCs with CCAs, or bile duct malignancies, accounting for 10-15% of the remaining tumours.

In the UK PLCs are the 18th most common cancer type and account for 2% of new cancer diagnoses annually (Cancer Research UK, 2017). However, it is predicted that age standardised rates (ASR) of British PLCs will increase, by up to 43% for men, between 2014 to 2035 from 16 to 23 cases per 100,000 (Smittenaar et al., 2016). The predicted rise in cases for women is less marked, from 6.87 to 8.32 cases per 100,000 patients within in the same timeframe, but is nonetheless a notable increase in disease frequency. Therefore, there is a national (and international) need for improved understanding of the mechanisms involved in tumourigenesis for both current and future patients.

The most pronounced increases in rates of PLC have generally been observed in Holland, the UK and the USA (Liu et al., 2019). The reasons for this are multitudinous and centre on the fact that the aetiology of primary liver is evolving from traditional risk factors (viral hepatitis & alcohol - discussed more below) to include newer aetiologies (obesity, type two diabetes and the metabolic syndrome) that are only now becoming clinically apparent. These conditions result in chronic liver damage and cellular regeneration and can result in the reversible deposition of fibrous tissues (fibrosis) upon

repeated injury-regeneration cycles that can progress to the permanent deposition of scar tissue (cirrhosis). The correlation between fibrosis, cirrhosis and carcinogenesis has been established in HCC but does not correlate with rates of cholangiocellular carcinogenesis, as most of these tumours occur sporadically.

1.1 Hepatocellular Carcinoma

The World Health Organisation (WHO) classify seven subtypes of Hepatocellular Carcinoma, based on morphological phenotypes (*International Classification of Diseases for Oncology*, 2019). The tumour subtypes are: hepatocarcinoma, fibrolamellar, scirrhous (sclerosing), spindle cell variant (sarcomatoid), clear cell, pleomorphic and mixed (hepatocholangiocarcinoma/ HCC-CCA) tumours.

Other classifications are available such as those based on cellular architectural patterns or cytological variants and can occur in combination (Hamilton & Aaltonen, 2000; Torbenson, 2017). Architectural variants include: trabecular (plate like), pseudoglandular/ acinar, compact and scirrhous. Cytological properties include: pleomorphic cell, clear cell, sarcomatous change, fatty change, bile production, inclusion bodies (Mallory hyaline, globular hyaline, pale or ground glass), fibrolamellar and finally undifferentiated HCC (Hamilton & Aaltonen, 2000). Of note, where the tumour may display characteristics from more than one morphological subtype, the tumour with the worse prognosis is ultimately used for classification and prognostication (Torbenson, 2017). Some variants of HCC – such as scirrhous – have no prognostic difference when compared to classical HCCs (Jiang et al., 2018). However, prognostic differences between HCC subtypes, such as fibrolamellar and mixed tumours have a better and worse prognosis respectively (Bergquist et al., 2016; Jiang et al., 2018; Torbenson, 2017). Hence, HCC classifications are not exhaustive, or mutually exclusive, and cannot possibly reflect the wide variety of genetic mechanisms involved in carcinogenesis. There are clearly many ways of sub-classifying HCCs, some

of which will not have been recorded in archived clinical samples. For the purposes of consistency, the WHO adopted classification will be used in preference to others.

Overall, there is a significant volume of evidence describing tumour morphology in great detail with mixed evidence for patient outcomes, such as overall mortality. In the era of personalised medicine, with a multitude of aetiological causes of PLC the genetic make-up of tumours may hold the key to further understanding neoplastic initiation and development.

1.1.1 Subtypes

1.1.1.1 Good prognosis: FL-HCC

A relatively common subtype of HCC that occurs in younger patients and carriers a more favourable prognosis is called fibrolamellar HCC (FL-HCC). Intra-tumoural bands of parallel fibrous tissues are characteristic for this disease that is diagnosed based on typical histological characteristics. Contrary to traditional HCCs, fibrolamellar tumours are only found in patients where there is no background cirrhosis present (Torbenson, 2017). There are also no known risk factors for developing FL-HCC (Shafizadeh & Kakar, 2013) but a segmental deletion yielding the DNAJB1-PRKACA gene fusion has been recognised as a signature event in FL-HCC, (Kastenhuber et al., 2017).

1.1.1.2 Intermediate prognostic tumour types

The so called ‘classical HCCs’ account for the majority of hepatocellular tumours with no difference in prognosis between classical and scirrhous subtypes (Jiang et al., 2018). The steatohepatitic variant of HCC is, somewhat unsurprisingly, associated with metabolic diseases but does not appear to have any correlation with gender, aetiology, tumour size, number of lesions or grade of tumour (Jain et al., 2013). In a small HCV cirrhosis associated HCC cohort, 35% of HCCs were of the steatohepatitic subtype,

with 64% of this subgroup having at least one risk factor for NAFLD/ NASH (Salomao et al., 2010).

Clear cell HCCs are generally well differentiated tumours arising on a background of cirrhosis, and have a male preponderance (2.3:1, M: F ratio) but are more common in women than traditional HCC (~7:1 M: F ratio) (Yang et al., 1996). There is mixed evidence showing either slightly better, or equivocal, differences in prognosis of clear cell HCCs (Jabbour et al., 2019; Yang et al., 1996). Clear cell HCCs carry a higher rate of isocitrate dehydrogenase 1 (IDH-1) mutations compared to other HCC subtypes with these mutations conferring a poorer prognosis by altering cellular metabolism and promoting tumourigenesis (Lee et al., 2017). This molecular sub-classification of tumours holds intriguing diagnostic and therapeutic implications as the advent of targeted IDH therapies may increase the therapeutic arsenal available to clinicians.

Scirrhous HCCs highlights the diagnostic difficulties of assessing PLCs, as they can be radiologically indistinguishable from CCA, whilst also possessing intra-tumoural fibrosis similar to FL-HCC (Jabbour et al., 2019). However, when assessing immunohistochemical expression, a combination of Arginase-1 and Glypican-3 (GPC-3) expression is 100% sensitive for scirrhous HCC when differentiating from CCA (Krings et al., 2013). This is further evidence of the power of specific, targeted, analysis in PLCs, and that different approaches may yield similar results for tumour assessment and stratification.

1.1.1.3 Poor prognostic tumours

Mixed HCC-CCA, Sarcomatoid and small cell HCCs are quite rare and hold a poorer prognosis compared to classical HCCs. In many respects, sarcomatoid HCCs have similar properties to mixed HCC-CCA, with comparable rates of lymph node metastases and adjacent organ invasion (Li et al., 2018). In terms of median overall

survival, sarcomatoid HCCs have a worse prognosis (8.7 months with a 0% 3-year survival) compared to mixed tumours (24.9 months, 36% 3-year survival) (Li et al., 2018; Liao et al., 2019). If patients undergo liver transplantation, mixed tumours have a high rate of recurrence, especially within the first year after surgery, demonstrating their aggressive tumour behaviour (Park et al., 2013).

The rarity of these subtypes of HCC makes estimates of prognostic differentiation challenging this is typified by case reports identifying small cell HCCs, a case series of three tumours (from 520 HCCs at one centre) described poor survival after diagnosis (one month for two patients and 5 months for the third patient) in elderly patients (Zanconati et al., 1996). Two of these patients were deemed unfit for invasive treatment and the tumours were found at autopsy.

1.1.2 Aetiology

Around 90% of HCCs arise in the context of chronically inflamed, scarred, cirrhotic, liver tissues (Jindal et al., 2019; Mittal & El-Serag, 2013). The inflammation-metaplasia-carcinoma sequence that occurs is responsible for the majority of these cancers developing as the increased rates of cellular turnover hence allowing more opportunities for driver mutations to develop. However, there are certain circumstances when tumours occur in the absence of cirrhosis, such as in the context of NAFLD without cirrhosis and in fibrolamellar tumours (Kulik & El-Serag, 2019; Torbenson, 2017).

Globally, the rates of PLC are changing to reflect chronological variations in causative risk factors – with reductions in countries where Hepatitis B Virus (HBV) was previously a major driver (SE Asia and Africa) and rising incidence in countries like the UK, which have seen a sharp increase in number of individuals with cirrhosis.

However, the legacy of chronic viral hepatitis still accounts for the majority of HCC cases, when considered at the international scale.

The global burden of diseases such as hepatitis, cirrhosis and diabetes have increased between 1990 - 2010 by 46.4%, 32.5% and 92.7% respectively (Lozano et al., 2012). These deeply concerning increases in liver cancer-associated conditions go some way to explain the recorded increase in rates of PLC. Western diet and lifestyle will play an increasing prominent role in the aetiology of future PLCs in North America and Europe. As obesity rates continue to soar in the Western world, future rates of PLC are predicted to reflect the already measurable increase in obesity driven cancer development (Massoud & Charlton, 2018).

1.1.2.1 Fibrosis & Cirrhosis

Scarring of the liver (cirrhosis) is an irreversible change that results from chronic inflammation and is found in 80-90% of post mortem autopsies in patients with HCC (Fattovich et al., 2004). The majority of the remaining 10 – 20% of HCCs in this study had a degree of liver fibrosis with only a very small proportion having absolutely normal background liver histology (Fattovich et al., 2004). More recent evidence has indicated that the risk of HCC remains low in all patients without cirrhosis, with the exception of those with higher fibrotic indices (Kulik & El-Serag, 2019). However, there is no simple dose-response relationship for fibrosis/cirrhosis and HCC development, indicating the complexity of this highly heterogeneous disease entity. Amongst newly diagnosed cases of HCC, in the absence of advanced fibrosis or cirrhosis, NAFLD is the largest aetiological factor in an American study cohort (Kulik & El-Serag, 2019).

Fibrosis is the result of increased extra cellular matrix (ECM) deposition secondary to chronic inflammation and activation of both the innate and adaptive immune responses

(Parola & Pinzani, 2019). The pro-fibrogenic environment resulting from chronic inflammation leads to hepatic stellate cell (HSC) recruitment, activation and trans-differentiation into fibrogenic myofibroblasts (Tsuchida & Friedman, 2017). Inhibiting the activation of HSCs is the target of a number of phase II and III clinical trials which are exciting potential therapies of the future. As the number of hepatotoxic insults increases the risks of developing HCC is also raised. An example of multifactorial hepatotoxic insults is highlighted by the increase in incidence of HCCs in non-cirrhotic diabetics who drink alcohol, owing to the synergistic effect of multiple hepatotoxins (Kulik & El-Serag, 2019).

Changes in hepatic cellular structure and physiology from ECM deposition, proliferating activated stellate cells and neovascularisation of hepatic sinusoids result in cirrhosis (Tsochatzis et al., 2014). Cirrhosis was previously considered an advanced stage of liver fibrosis with irreversible distortion of the hepatic vasculature (Schuppan & Afdhal, 2008). However, there is increasing evidence from the follow up of treated viral hepatitis patients, that this might not be the case. Successful treatment of the underlying pro-fibrotic disease reduces the pro-inflammatory environment and leads to a regression in fibrotic indices as fibrolytic pathways are up-regulated (Jung & Yim, 2017).

When the native sinusoidal endothelial cells are disrupted, there is shunting of blood from portal and arterial vessels to the hepatic veins results in a decrease in exchange between hepatic sinusoids and hepatocytes, further reducing liver function (Schuppan & Afdhal, 2008). The molecular mechanisms behind this are complex, but sinusoidal endothelial cells are defenestrated then *capillarised*, causing a loss of substrate exchange (Zhou et al., 2014). As the functionality of liver cells decreases with repeated exposure to noxious stimuli, scar tissue deposition increases further reducing the functional capacity of the liver (Jung & Yim, 2017).

Annual rates of HCC incidence in cirrhosis varies between 4 – 30%, depending on the underlying aetiology (PBC and HCV respectively), demonstrating an irrefutable increase in cancer-risk, regardless of the underlying disease process causing cirrhosis (Fattovich et al., 2004).

1.1.2.2 Infectious Causes of HCC

Globally, HBV and HCV are thought to be responsible for 43.3 % and 18.7% of primary liver cancers respectively (Liu et al., 2019). The prevalence of these infectious diseases, and their legacy (i.e., cirrhosis and liver cancer) varies widely depending on geographical location. Liver cancer has traditionally been most prevalent in eastern Asian countries and sub-Saharan Africa, and between them account for up to 83% of cases globally (GLOBOSCAN 2012 (IARC), 2015). In the most part this is due to vertical transmission of HBV during childbirth coupled with historically low rates of vaccination against HBV in these host countries. The risk of developing HCC from HBV is associated with the length of infection, viral load and the severity of liver disease present as HBV is able to integrate into the hosts' DNA (Forner et al., 2018; Schulze et al., 2015).

The other main hepatotropic infection, HCV is more prevalent in Western populations and are associated with iatrogenic infection from blood product transfusion or recreational intravenous drug use (El-Serag, 2011; Forner et al., 2012). HCV is now curable in over 95% of cases with the direct acting antivirals (DAA) and the widespread use of these has already resulted in a fall in number of cases of chronic HCV, and fewer cases of HCV-associated HCC.

However, active viral hepatitis remains the main driving factor behind global HCC development (Kulik & El-Serag, 2019). As such the WHO has invested heavily in a

global effort to eliminate viral hepatitis transmission in all member states, with an ambitious target of a 90% reduction in new cases by 2030 (Cooke et al., 2019).

In the US, prior to curative treatment for HCV being available, rates of cirrhosis had doubled in patients with HCV along with a 10-fold increase in the rates of HCC (Mittal & El-Serag, 2013). A demonstration of the dynamic relationship between aetiology and carcinogenesis is exemplified by rates of HCV attributable HCC that are decreasing, after the advent of direct acting antivirals (DAA), from 46.4% in 2013 (prior to DAA use) to 33.7% in 2016, when measured by transplant-indication for HCC treatment (Vaziri et al., 2018). Age standardised rates of PLC due to HCV had increased by 7.6% from 1990 to 2010, whilst those due to HBV have only risen by 2.6% in the same period (Lozano et al., 2012). With synergistic effects of multiple hepatotoxic insults on fibrotic and cirrhotic changes reducing the local and global burden of HBV and HCV infections will lead to a decrease in future HCC development (Jindal et al., 2019; Mittal & El-Serag, 2013).

We very much find ourselves in unknown territory for monitoring patients who have achieved a sustained viral response (SVR) against HCV, as a result of DAA therapies. Previously these patients would have required regular surveillance for HCC development as they were deemed high risk but the need for large prospective multicentre studies are required to clarify this (Peck-Radosavljevic and Singal, 2019).

In SE Asia and sub-Saharan Africa, a carcinogen produced by *Aspergillus* species (Aflatoxin B1) is able to grow in foodstuffs (grain, corn, cassava) when stored in high moisture conditions (Mittal & El-Serag, 2013). The dietary ingestion and hence exposure to these toxins promotes mutations in the tumour suppressor gene, p53, resulting in a genetic basis of tumourigenesis in as many as 90% of Aflatoxin-related HCCs (Gouas et al., 2009). The regions where Aflatoxin-related HCCs and HBV-related HCCs overlap considerably account for a synergistic effect of dual-exposure

leading to an increased chance of developing HCC (Schulze et al., 2015). Fortunately, rates of Aflatoxin-related PLCs in the West are vanishingly rare as the climatic conditions required for *Aspergillus* growth simply do not occur.

1.1.2.3 Diet, Lifestyle & Environmental.

The link between heavy alcohol consumption (>80g/day) and cirrhosis development is well established, however full comprehension of the mechanisms involved remain incompletely understood (Mittal & El-Serag, 2013; Morgan et al., 2004). What is clear is that even mild alcohol consumption (10g/day, ~ 1unit) is associated with a higher rate of carcinogenesis (Ganne-Carrié & Nahon, 2019). The mechanisms whereby alcohol induces changes in cellular signalling are complex. Increased production of reactive oxygen species, increased gut permeability, changes in folate and lipid metabolism, activation of innate immunity and toxic effects on mitochondrial biology are just some of the mechanisms proposed to be adversely altered by ethanol (Ganne-Carrié & Nahon, 2019). The societal effects of chronic alcohol ingestion (addiction, unemployment, tobacco smoking etc.) and those in lower socioeconomic groups are associated with poorer outcomes. Those addicted to alcohol present at a later stage, have poor concordance to screening and follow up owing to the addictive nature of the substance (Ganne-Carrié & Nahon, 2019).

In Italy and the US, alcohol is thought to cause between 32-45% of HCCs, and at higher rates when there was HCV was a cofactor in tumour development (Morgan et al., 2004). Globally alcohol accounts for 14.7% of primary liver cancers in 2016, but there are wide geographical variations with up to 34.5% of PLCs in Western Europe being attributed to alcohol excess (Liu et al., 2019).

As waistlines expand and medical conditions such as dyslipidaemia, diabetes mellitus and the metabolic syndrome are increasing due to poor diet and an increasingly

sedentary lifestyle. These metabolic diseases are resulting in increased rates of primary liver cancer owing to the metabolic burden being placed on the liver as it digests the increased dietary fat and macronutrients that result in the inflammatory states: non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis: NASH (Jindal et al., 2019). Diet and lifestyle factors are inextricably linked to type two diabetes, the rates of which are increasing globally. Whilst type two diabetes is a risk factor for HCC development, there is also the possibility of NAFLD acting as an independent risk factor, though the evidence for this is less certain (Kulik & El-Serag, 2019). Rates of death from PLC are increased five-fold in those with a high baseline body mass index (BMI), further highlighting the link between obesity and hepatocarcinogenesis (Forner et al., 2018). Regardless of geographical location, alcohol intake or diabetic status, an elevated BMI (>25 kg/m^2) is linked to liver cancer generation (Jindal et al., 2019).

1.1.3 Incidence

A national study, using data from all hospital discharge records in France from between 2009 and 2012 found 31927 incident cases of adult HCC giving an annual incidence of 16.4 cases per 100,000 (Goutté et al., 2017). Eighty percent of these cases were in men, 73.4% occurred in cirrhotic patients and 44% were as a result of alcohol consumption, in the absence of viral infection. Somewhat surprisingly, 36.4% had an unknown aetiology.

Incidence rates have exploded in Western populations recently, with a rise of 62.8% of cases in men (41.2% in Women) between 1990 to 2009 in the US (AACR Cancer Progress Report Writing Committee et al., 2013). Furthermore, in America, HCC cases have increased considerably since the 1970's. In 1973 the incidence of HCC was 1.51 cases per 100,000 whilst in 2011 it was 6.20 per 100,000 (Njei et al., 2015). Interestingly, there have been large increases in PLC in high socioeconomic index countries, with the UK having the third highest increase in estimated annual percentage

change, reflecting both the changing landscape of aetiological conditions (amongst others: rich diet and alcohol as mentioned above) associated with PLCs, (Liu et al., 2019).

Gender is important in HCC development and varies geographically with a 4:1 male: female ratio in South Korea, and approaching a 2:1 (male: female) ratio in UK (Mittal & El-Serag, 2013). Circulating oestrogen plays a role in HCC tumour development as fewer women develop HCC, and in women who are treated for HCC, oestrogen replacement (for menopausal symptoms) is protective, giving a reduced risk of HCC-recurrence and prolonged survival (Fujiwara et al., 2017). The immunomodulatory effects of oestrogens (immune-stimulating) is thought to result in less inflammatory damage, apoptosis and oxidative stress compared with immune suppressing androgens which increases fibrogenesis (Ruggieri et al., 2018).

However, developing countries may be underestimating the reported rates of HCC owing to their poor-quality data provided, and limited abilities to detect or manage HCCs, with an estimated 120,772 missed incident cases of HCC in 2012 (Sartorius et al., 2015). This incomplete data makes accurate estimates of HCC rates difficult to fully describe, but are improving with time.

1.1.4 Stage

Numerous disease staging systems used when assessing HCCs. These include the tumour- lymph node-metastasis (TMN), Okuda, Cancer of the Liver Italian Program (CLIP), Japanese Integrated Staging (JIS), Chinese University Prognostic Index (CUPI), Hong Kong Liver Cancer (HKLC) and most commonly used in the West, the Barcelona Clinic Liver Cancer (BCLC) staging system (Forner et al., 2018). HCC is stage-orientated for cancer therapies, with various treatment options offered to patients depending on the *stage hierarchy* of their disease (Vitale et al., 2020). These stage-

boundaries are not fixed, as roughly half of patients benefit from down-staging therapies able to reduce tumour burden, meaning carefully selected patients *can* be considered for curative treatment such as liver transplantation (Bryce & Tsochatzis, 2017). Staging disease is currently based on radiological and liver dysfunction parameters, with minimal emphasis being placed on individual tumour biology in this era of personalised medicine. The one exception being serially measured alpha-foetoprotein (AFP) >100ng/ml was found to be the only independent risk factor of HCC recurrence post liver transplant (Mehta et al., 2020).

Generally, in earlier stage disease more treatment options are available to patients. The five BCLC stages of HCC (stage 0, A, B, C and D) and their respective survival statistics are outlined in figure 1, which takes into account not only the stage of disease, but also other disease characteristics (such as the size and number of tumours, portal pressures, liver function and other associated diseases) as well as the patients performance status (Forner et al., 2018).

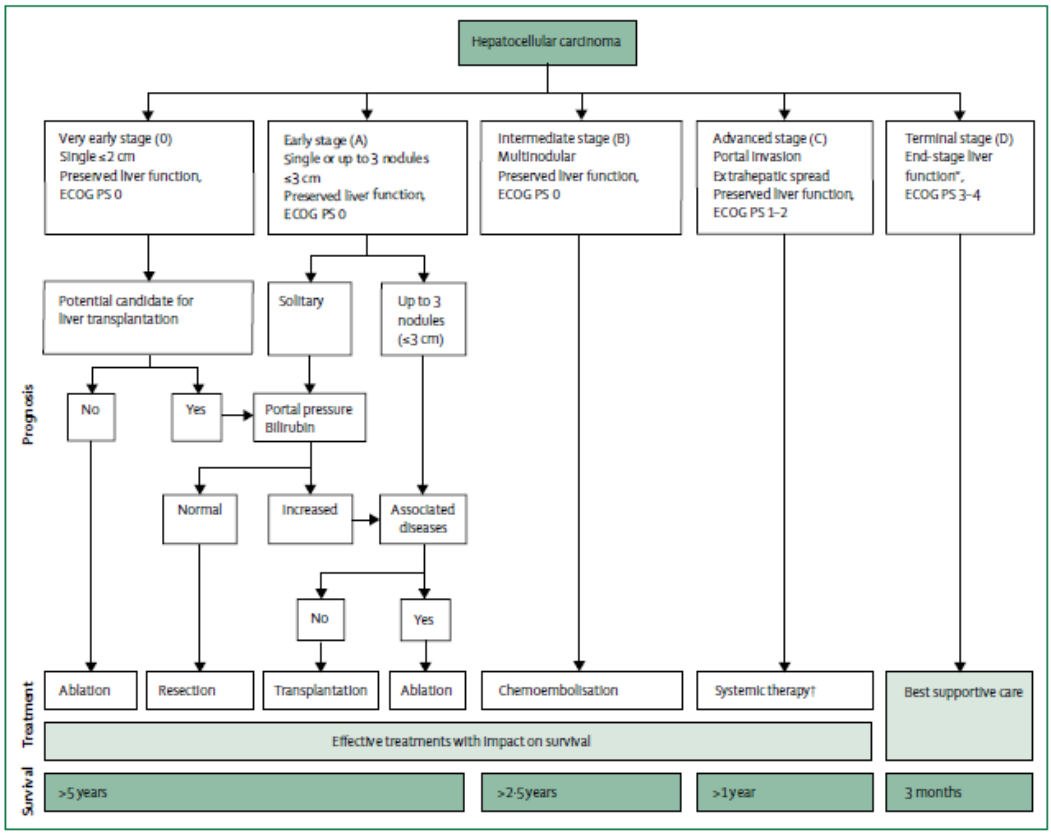


Figure 1. Stages of HCC disease, management and expected survival.

Therefore, patients that undergo surgical resection, or transplantation, have relatively confined tumour disease, which has the potential to be a selection bias to any studies solely analysing these tissues.

1.1.5 Grade

Since 1954 the Edmondson-Steiner (ES) classification of HCCs has been in use with grades I-IV correlating with adverse features of cellular differentiation (Edmondson & Steiner, 1954). From the most differentiated (grade I, well differentiated), to increased cytoplasmic & nuclear volumes (grade II, mild differentiation), increased hyperchromatic features with an increase in relative nuclear proportion in the cell (grade III, moderately differentiated) to variable, often scanty, cytoplasmic volumes with intensely abundant and hyperchromatic nuclei and non-cohesive cell masses (grade IV, poorly differentiated) (Nakashima et al., 1987). This four-tier system has not always been adopted, with a modified three-tier (well, moderately and poorly differentiated)

grading system occasionally used (Rastogi, 2018). With ES' grades I and II being incorporated into a modified 'well differentiated' class to facilitate this simplification, but grouping tumours as grades I & II (favourable) vs III & IV (unfavourable) has been undertaken extensively in the literature (Pawlik et al., 2007).

Interestingly, whilst pre-operation biopsy of tumour samples has no correlation with post-transplant survival, comprehensive histological assessment of explant livers does give an accurate determinant of survival post liver transplant, with worst outcomes in the poorest differentiated tumours (Pawlik et al., 2007; Tamura et al., 2001). This may well confirm that comprehensive assessment of the whole tumour is required to reduce the sampling error that inevitably occurs from a biopsy. Tumour stage remains the most important factor when assigning appropriate treatment regimens in HCC.

1.1.6 Regional Variations in HCC Prevalence

Regional variations in HCC broadly reflect the differences in aetiology underlying carcinogenesis. South East Asian, Chinese, Sub-Saharan and East-African cancers account for >80% of the number of cases and are predominantly due to HBV and, to a lesser extent, Aflatoxins (Choo et al., 2016). The vertical transmission of HBV results in HCC diagnosis approximately a decade younger than those from North America and Europe, where HCV is acquired later in life (Mittal & El-Serag, 2013). Mittal & El-Serag also state that alcohol consumption, HBV and HCV rates are higher, and potentially more carcinogenic, in men which may partially explain the male preponderance of HCCs. Zucman-Rossi et al have grouped these more aggressive tumours (HBV-associated, increased vascular invasion, poorer overall survival) together and called them a *proliferation class*, with the *non-proliferation class* being made up of HCV and alcohol-related disease which confers a less aggressive phenotype (Zucman-Rossi et al., 2015). Alongside viral hepatitis, NAFLD is another common disease entity that is becoming increasingly responsible for both Eastern and Western HCC

development (Choo et al., 2016). In America, NAFLD is now the leading cause of chronic liver disease (Mittal & El-Serag, 2013).

In the UK HCC mortality rates are steadily increasing, compared to the overall downward trend in both European neighbours and across the world. In the UK both men (+67.9%) and women (+57.1%) saw significant increases in mortality rates, compared to France (M: -14%, F: -12.6%), Germany (M: +15.6%, F: +1.3%) and Spain (M: -14.7%, F: -39.2%) as well as significant decreases in Japan (M: -42.1%, F: -41.0%), Korea (M: -30.1%, F: -29.0%) and Hong Kong (M: -23.7%, F: -11.0%) thus highlighting global differences in HCC mortality data (Bertuccio et al., 2017). With such marked increases in UK HCCs, further work describing this cohort is vital to help improve patient outcomes.

Owing to the global variations in the aetiology of HCC, Zucman-Rossi state: "the highly heterogeneous nature of HCC makes genomic classification of this disease and identification of clinically relevant biomarkers more challenging compared with other solid tumours like lung and colon cancers" (Zucman-Rossi et al., 2015).

1.1.7 Genetics of HCC

The genetic landscape of HCC is incompletely understood. Large multicentre studies of European patients with HCC are based on French and German patient cohorts (who also have a decreasing HCC-specific mortality rates, contrary to the UK) and are based on surgically resected tumours. The ability to sequence the exome, transcriptome and whole genome has allowed genetic variations, their associations with nucleic acid sequences and ultimately altered protein expression, to be correlated with disease characteristics. Cell signalling pathways involved include altered telomere biology, cell cycle, Wnt/beta-Catenin, epigenetic, NRF2/KEAP1, RAS/ RAF/MAPK and AKT/mTOR pathways (Madduru et al., 2019; Nault et al., 2020).

Driver mutations occur when random genomic alterations are clonally expanded because they have occurred in a 'cancer gene' (Hanahan & Weinberg, 2000; Stratton, 2011). The neoplastic cells then have a survival advantage because of their increased ability to divide, can resist normal homeostatic control or otherwise demonstrate a hallmark of cancer. If a mutation does not confer a survival benefit, which occurs in the majority of cases, then these are termed passenger mutants (Stratton, 2011).

Enormous effort researching the genetics of HCC has been undertaken using fresh/frozen tumour samples from surgical resection specimens and discovered that Telomerase activation is the most common mechanism for tumour initiation in HCCs, occurring in 70-90% (Nault et al., 2019). Recruitment for these studies has so far not included UK-based patients to any significant degree. Telomerase promoter mutations ($pTERT^{Muts}$) are associated with alcohol driven HCCs in a western European cohort, indicating a potential aetiology-specific association (Schulze et al., 2015). Other groups have found an association between HCV-driven HCCs and $pTERT^{Muts}$, demonstrating that there is incomplete understanding of the exact mechanisms involved in telomere biology and carcinogenesis (Ally et al., 2017). It is clear that more work is required to improve understanding of any potential associations.

However, the available data provides an intriguing insight into how genetic alterations are associated with aetiological processes. Early work described the role of tumour protein 53 (TP53) mutations in Aflatoxin B1 associated HCCs and subsequent understanding of other cell-signalling pathway alterations have been facilitated by the technological advances (Nault et al., 2020).

With the increased availability of next generational sequencing technologies, subgroups of HCCs have been found based on their genetic profiles. For HCCs the most commonly dysregulated driver pathway has been in aberrations in the Telomerase Reverse Transcriptase (TERT) gene occurring in 70-90% of all tumours (Nault et al.,

2019; Schulze et al., 2016). This Telomerase enzyme is involved in altered Telomere maintenance via promoter mutations (60%), gain of function amplifications (5%) and Hepatitis B Virus (HBV) integration (5%). Other candidate drivers that have been described include TP53, CTNNB1 and ARID1A (Schulze et al., 2015; Totoki et al., 2014). These studies have focussed on fresh-frozen libraries of liver tissues, owing to the relative ease of using this resource when compared to FFPE archives. Further evidence of telomere biology alterations in liver disease has been shown with shorter telomere length and rare promoter mutations associated with NAFLD (Donati et al., 2017).

Recently irregularities in the diagnostic accuracy of cellular morphology alone for detecting fibrolemellar HCC have been highlighted, with some inter-reporter inconsistencies becoming apparent. Protein kinase cAMP-activated catalytic subunit alpha (PRKACA) FISH assays for a genetic rearrangement were positive in 99% (102 of 103) of typical FL-HCC, and in 75% (9/12) of the diagnostically uncertain (initially categorised as '?FL-HCC') when assessed with cellular morphology alone (Graham et al., 2018). Some tumours that are initially misdiagnosed as HCC later turn out to be FL-HCCs containing the gene fusion when appropriately analysed, demonstrates the power of assessing a single genetic abnormality (Lalazar & Simon, 2018; Simon et al., 2015).

1.1.7.1 UTAA in HCC

Survivin and Telomerase have been described as the archetypal universal tumours antigens (UTAA) as they are expressed in a wide range of cancers and are a *Hallmarks of Cancer* (Hanahan & Weinberg, 2000, 2011). When any tumour expresses UTAA they are deploying mechanisms to evade normal physiological control by evading apoptosis (Survivin) or enabling replicative immortality (Telomerase) to promote tumourigenesis.

Survivin predominantly present in the nuclei of HCC tumours with IHC and correlates with proliferation indices (Ito et al., 2000). Indeed, it is the nuclear translocation of Survivin in HCCs from the cytoplasm of quiescent non-malignant liver cells that may constitute an important mechanism of cell proliferation and differentiation in carcinogenesis (Moon & Tarnawski, 2003). Significant positive correlations exist between IHC-detected nuclear Survivin and grade, microvascular invasion, mitosis, local recurrence and disease-free survival indicating the essential role Survivin may play in hepatocarcinogenesis (Fields et al., 2004).

Telomerase activity is present in around 90% of HCCs, with promoter mutations being both responsible for this, and the most common genetic alteration in HCC (Nault et al., 2019). Indeed, these promoter mutations are HCC specific, with none occurring in CCA (Quaas et al., 2014). Telomerase activity, as measured by the telomerase repeat amplification protocol (TRAP assay – a PCR based assessment of enzyme activity) has been linked to an increase risk of tumour recurrence after resection, suggesting a role in malignant tumour biology (Kobayashi et al., 2001). Telomerase protein, detected by IHC, in tumour tissues has been linked to tumour worse grade, the presence of a portal vein thrombosis and HBV insertion into the genome in a Chinese cohort (X. Zhou et al., 2016). Whilst serum detection of telomerase mRNA has been proposed as an adjunct to AFP to increase the accuracy of HCC detection (El-Mazny et al., 2014).

1.1.8 Treatment

The treatment of HCC is complex and determined by tumour stage and underlying liver disease severity. There is a deficit of care between the most unwell patients (advanced tumour burden, advanced liver cirrhosis and poor liver function) and the treatment options they are offered. In simple terms: the patients most in need of therapy are not medically fit for surgical resection, and all treatment is therefore palliative in nature.

Curative options are more likely to be successful in early disease and in the UK, many centres use the Barcelona Clinic Liver Cancer (BCLC) staging of disease to guide treatment decisions (Llovet et al., 1999). Criteria that are measured to facilitate the BCLC stage are the size of the tumour, the presence and severity of cirrhosis and other markers of liver functionality as well as the patients' ability to perform their activities of daily living (Hartke et al., 2017).

1.1.8.1 Surgical.

Surgical management offers the best chance of a cure for HCC, but is only offered to 5% of patients based on their stage of disease at diagnosis (El-Serag, 2011). In the absence of cirrhosis, surgical resection of a portion of the tumour containing liver is the treatment of choice with long term cure possible (Forner et al., 2012).

Liver transplantation is an option for some cases and offers the prospect of curing both the HCC and the underlying liver disease. However, the risk of tumour recurrence post-transplant increases with tumour size and decisions are often based on the internationally recognised Milan criteria (C.-Y. Liu et al., 2015; Mazzaferro et al., 1996). Patients with early-stage disease (BCLC stage A) defined by a single tumour being 5cm or less, or up to three tumours with the largest 3cm or less, with no vascular spread or extra-hepatic disease in the context of moderate to severe cirrhosis benefit the most from liver transplantation (Galle et al., 2018; C.-Y. Liu et al., 2015). Only a small minority of patients fulfil these criteria leaving the majority of patients' very limited options, most of which are often palliative.

1.1.8.2 Loco-regional therapies.

Transarterial chemoembolisation (TACE) therapy involves the delivery of chemotherapy via a catheter to a vascular tumour. Conventional TACE uses a Lipiodol based emulsion to deliver a chemotherapeutic and embolising agents, whereas Drug

Eluting Bead TACE (DEB-TACE) uses chemotherapy loaded beads delivered into the tumour vasculature to release antineoplastic agents over a longer period of time whilst also embolising the tumour's arterial supply (Raoul et al., 2019). TACE has become the treatment of choice in intermediate stage HCC as it is a life prolonging treatment but it can also be used as a down staging (bridging) therapy to reduce tumour size and facilitate liver transplantation.

TACE has been quoted as achieving a successful downstage in 48% of cases with a tumour recurrence rate in the transplanted liver around 16% when reported in a recent meta-analysis of 950 patients (Parikh et al., 2015). There is no benefit of neo-adjuvant Sorafenib (a multi-kinase inhibitor) plus TACE compared to TACE alone prior to liver transplant for HCC, as patients' tumours from the two groups demonstrated similar time to progression (Hoffmann et al., 2015).

When not prescribed as a bridging therapy TACE is the most commonly used palliative treatment regimen when surgical treatment is not possible. For treating intermediate stage (BCLC stage B) HCC, DEB-TACE confers a median survival of 47.7 months when BCLC treatment guidelines are strictly adhered to (Burrel et al., 2012). However, it is acknowledged that these inferences can only be made when there is judicious use of BCLC criteria. TACE can be used out with intermediate stage disease, but less strict adherence to BCLC guidelines can mean patient outcomes may be compromised (Palmer et al., 2020; Raoul et al., 2019).

Combining systemic therapies with DEB-TACE (Sorafenib and DEB-TACE) in intermediate (BCLC stage B) HCC demonstrates no reduction in time to progression in the dual treated arm of the trial when compared to DEB-TACE alone (Lencioni et al., 2016). Further studies are on-going, exploring the role of immunotherapy being used in conjunction with loco-regional therapies, with initial results demonstrating combined

therapies are well tolerated, but significantly more work in this area is required (Greten et al., 2019).

1.1.8.3 Ablative Treatment

Radiofrequency ablation (RFA) and microwave ablation (MWA) are physical treatments that lyse cells by generating heat. The ablation is targeted using ultrasound or CT and a probe inserted into the tumour to deliver the thermal injury. RFA is able to deliver complete tumour necrosis and has similar outcomes compared to surgical resection in BCLC stage 0 (very early stage, single nodule tumour < 2cm) HCC, at a reduced cost (Galle et al., 2018). This study compared 3996 resection and 4424 ablated patients and concedes that the only benefit of surgical resection would be to provide tissue for histopathological analysis of high-risk features, such as vascular invasion, poor differentiation and satellite tumours. Judicious use of RFA in a clinically appropriate patient can be used in conjunction with surgical resection in intermediate (BCLC B, normally treated with locoregional therapy alone) stage HCC, demonstrating a combination of chemotherapeutic regimes may lead to better overall survival with a longer time to progression (Espinosa et al., 2018).

1.1.8.4 Systemic therapies.

First line treatment for advanced HCC (BCLC stage C) has been the multi-kinase inhibitor, Sorafenib, for more than a decade (Bouattour et al., 2019). Sorafenib targets tyrosine kinases, significantly improves overall survival from 7.9 to 10.7 months and results in a longer time to progression (5.5 vs. 2.8 months) when compared to placebo (Rimassa et al., 2019). Other immunotherapeutic options in HCC includes checkpoint inhibitors, currently offered after failed Sorafenib treatment, with further trials assessing the role of combination, or even first line use of this novel drug-class, (Onuma et al., 2020) .

With positive results emerging from phase II/III trials for second line agents including the multikinase inhibitors Regorafenib, Lenvatinib and Cabozantinib as well as encouraging results from studies of the check point inhibitors including Ramucirumab, Nivolumab and Pembrolizumab clinical guidelines will doubtless change very soon to reflect these developments (Bouattour et al., 2019; Foerster & Galle, 2019). Significant potential for further improving therapies targeting the most commonly altered genetic variations in HCC, such as $pTERT^{Muts}$, beta Catenin, TP53 and ARID1A driver mutations is highlighted by their presence in the majority of HCCs (Bouattour et al., 2019).

Clinically useful genetic aberrations that commonly occur in various tumours, including HCC, requires a 'drug-able target'. For TP53 this requires the wild type confirmation of p53 to be stabilised and allows the protein to fold normally. The ability to induce conformational change in p53 mutants by the compound APR-246 results in apoptosis induction in tumour cells and has been involved in clinical trials (Parrales & Iwakuma, 2015). The Wnt/beta-catenin pathway involves a large number of proteins and kinases with numerous targets being assessed in pre-clinical models and clinical trials. Various Tankyrase, Porcupine, Disheveled and beta-Catenin inhibitors are being researched in the pre-clinical setting (Krishnamurthy & Kurzrock, 2018). Further clinical trials exploring Porcupine inhibitors, Wnt antibodies, beta-catenin inhibitors, Notch signalling inhibitors, gamma secretase inhibitors and inhibitors of Hedgehog signalling are also being explored in a wide range of solid and haematological tumour types, further highlighting the potential for personalised medicine based on universally expressed tumour antigens (Krishnamurthy & Kurzrock, 2018).

For Telomerase, the oligonucleotide inhibitor, Imetelstat, targets the active site template region of TERC and demonstrates anti-tumour activity (Nault et al., 2019). Imetelstat is known to accumulate in the liver, spleen and bone marrow and has been used in phase

I/II trials for acute myeloid leukaemia and myelodysplastic disease (Saraswati et al., 2019). The small molecule Telomerase inhibitor, BIBR1532, has demonstrated positive pre-clinical results but further clinical studies are required (Tahtouh et al., 2015). Other examples of novel approaches for therapy includes immunotherapy targeted against Telomerase and Survivin. The multi-peptide vaccine strategies are in phase one trials for metastatic breast cancer (clinical trials identifier: NCT01660529) and advanced myeloma (clinical trials identifier: NCT00834665) with the results keenly awaited.

1.2 Cholangiocarcinoma

Cholangiocarcinomas (CCAs) are a wide-ranging and diverse group of malignancies that arise from biliary epithelial cells. Early work in this field did not differentiate between the different methods of classifying CCA, such as anatomical relationship, macroscopic growth pattern, microscopic features or classification by cell of origin (Krasinskas, 2018). As such there was significant room for improvement in international classification methods, and now a legacy of uncertainty regarding true rates of each tumour type. The most commonly used is the classification by anatomical site: intrahepatic, perihilar or distal (Krasinskas, 2018; Tyson & El-Serag, 2011; Wirth & Vogel, 2016). Confusingly the lack of consensus of nomenclature has resulted in a large number of approaches, with some authors using the intrahepatic/extrahepatic terminology when describing CCAs. As such there is an unreliable archive of classified tumours. Truly intrahepatic CCA (iCCA), are rare and account for roughly 10% of all CCAs, and 15% of PLCs. The majority of CCAs (~2/3) of extrahepatic cholangiocarcinomas (eCCA) are perihilar (pCCA) and the remaining ~1/3 are distal CCA (dCCA), but for convention both pCCA and dCCA have been grouped together as eCCA (Hemming, 2019, p219).

The nomenclature is important for differentiating CCAs as there has been a variation in reported use of terms used to describe the same disease entity that has lead to moderate

confusion and requires an international standard to be adopted to clarify this issue (Tyson & El-Serag, 2011). A specific example being the perihilar/ Klatskin/ extrahepatic tumour – which is the same disease by a variety of different names – that has variably been included with the intrahepatic CCAs, considered an entity in its own right and occasionally with other, more distal, cholangiocarcinomas. This is important to note as there have been reported surges in incidence rates of iCCA compared to eCCA, where the perihilar lesions are included with true intrahepatic CCAs (A. Bergquist & von Seth, 2015; Tyson & El-Serag, 2011). Accordingly, as many as 91% of perihilar tumours may have been erroneously recorded as iCCAs thus contributing to a false inflation in the incidence of iCCA when compared to other subtypes of CCA (Tyson & El-Serag, 2011; Welzel et al., 2007).

Intrahepatic cholangiocellular carcinomas arise from the second order bile ducts, or more peripherally within the liver parenchyma, with perihilar CCA originating from between the second order bile ducts and the cystic duct and finally distal CCAs are from the cystic duct to lower down the biliary tree (Banales et al., 2016). Other authors have concluded the differentiation between subtypes of CCA, in anatomical terms at least, as being in relation to the second order bile ducts alone differentiating between iCCA and eCCA (Krasinskas, 2018). There are ongoing efforts to standardise the diagnosis of CCA, based on anatomical location, despite the historical lack of clarity in this field.

Intriguingly the original classification of diseases in the ICD-0 and in the case of hilar CCA, have not been consistently recorded throughout recent history (Bridgewater et al., 2014). Leading to perihilar/hilar CCA coalescing independently with intrahepatic CCA and extrahepatic CCA across the published literature (Tyson & El-Serag, 2011).

Intrahepatic CCAs can be further sub-classified by their histological features and whether or not they are producing mucin (Krasinskas, 2018). Large duct, type 1, iCCAs tend to be columnar, produce mucin and resemble the bile duct epithelium whilst small

duct, type 2, iCCAs resemble cholangiocytes and are more cuboidal in their shape. Small duct iCCAs are associated with some risk factors shared with HCCs, such as viral hepatitis and cirrhosis and large duct iCCAs share aetiology with more chronic biliary diseases, which will be discussed further below. The type of tumour that grows tends to reflect the clinico-pathological features that are seen when comparing small and large duct neoplasms. Small ducts growths tend to be mass forming in nature and the larger ducts grow in a periductal infiltrating pattern, resulting in strictures or other thickened biliary epithelial growths (Banales et al., 2016).

1.2.1 Anatomical location

The nomenclature describing the site of CCAs relating to their anatomical site in relation to the cystic duct and the second order bile ducts, as outlined in figure 2, adapted from (Banales et al., 2016).

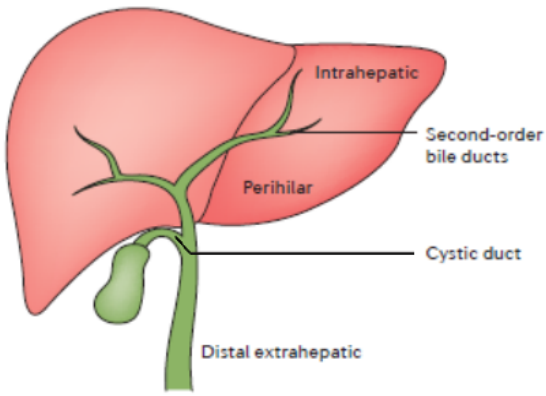


Figure 2. The anatomical locations of Cholangiocarcinoma.

Intrahepatic (also known as peripheral tumours) occur in the smaller calibre biliary tree, as denoted by the occurring further downstream than the second division in the bile ducts. Hilar/perihilar neoplasms occur between the second order bile ducts and the cystic duct, which receives bile from the gallbladder. Distal tumours occur further downstream than the cystic duct and can frequently present earlier with obstructive

jaundice-type symptoms. Prognosis is best for dCCA, moderate for pCCA and poor for iCCA in terms of overall survival and tumour-specific mortality (Hang et al., 2019).

1.2.2 Aetiology

Unlike HCC 70% of CCA arise sporadically, without pre-existing liver or biliary disease (Vogel & Saborowski, 2017). This is in stark contrast to HCC, where the majority of cases are in the chronically inflamed, cirrhotic, liver. The remaining ~30% of CCAs arise on the background of chronic biliary pathology, in the UK most commonly in patients with primary sclerosing cholangitis (PSC) (Tyson & El-Serag, 2011). The aetiology of CCAs can be broadly divided into sporadic, autoimmune, biliary, infectious, and environmental/ lifestyle associated.

Intrahepatic CCAs (iCCA) and HCCs can share various risk factors, such as HCV infection, non-alcoholic fatty liver disease (NAFLD) and hereditary haemochromatosis (HH) (Welzel et al., 2007). Extrahepatic CCAs are more associated with biliary disorders that impede the normal flow of bile such as primary sclerosing cholangitis (PSC), cholelithiasis (gallstone disease) and congenital abnormalities of the bile duct (Vogel & Saborowski, 2017).

Chronic inflammation of the biliary tree with biliary stasis, regardless of the underlying cause, is a significant risk factor when compared to a healthy population (Tyson & El-Serag, 2011). Examples of conditions that cause this include: biliary cirrhosis, cholelithiasis, hepatolithiasis, choledocholithiasis, cholecystitis and cholecystectomy (Tyson & El-Serag, 2011; Welzel et al., 2007).

Autoimmune conditions such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are associated with the development of CCA. PSC is most strongly associated with malignant cholangiocellular growth, with an astonishing 1560-fold increase in risk of developing CCA when compared to a background population (A.

Bergquist & von Seth, 2015; Wirth & Vogel, 2016). The lifetime risk of developing CCA in PSC is quoted at around 5 – 15%, but this may be an underestimation of the disease burden as post mortem diagnosis of CCA in PSC has been quoted to be as high as 41% (Levy et al., 2005). The annual risk of developing CCA in PSC is 0.5 – 1.5% with 50% of CCA tumours being diagnosed within one year of PSC diagnosis (A. Bergquist & von Seth, 2015).

When considering infectious causes as risk factors for CCA there is considerable geographic variation – one example being that 20% of Taiwanese having hepatolithiases, compared to 1-2% of patients in the West. The biologically plausible relationship between stone formation, chronic irritation and inflammation that results in malignant transformation appears sound. However, given that up to 30% of hepatolithiases are associated with liver fluke infections in endemic regions, these risk factors may not be independent as there may be a synergistic relationship between associated risks. The two main culprits for liver fluke infection are *O. viverrini* and *C. sinensis*, both of which come from raw, undercooked or pickled scaly fish from SE Asia with both flukes recognised as being carcinogenic to humans (Tyson & El-Serag, 2011). Fluke infection plays a significant role in CCA development in endemic regions but do not account for Western disease.

Other infectious risk factors associated with CCA development are the hepatitis viruses: HBV and HCV. The global burden of HCV is higher in Western countries, whilst HBV is more a disease of sub-Saharan Africa and South East Asia (Sia et al., 2017). Research into the role HBV and HCV play in iCCA development have unsurprisingly come to reflect the disease prevalence in the host country (Tyson & El-Serag, 2011).

As most cholangiocellular carcinomas are sporadic without apparent risk factors there are limited screening tools available (Banales et al., 2016; Khan et al., 2012). Around 30% of CCAs develop in patients with a chronic liver disease, such as PSC, and Khan *et*

al put it succinctly: “No benefit in screening for CCA in PSC has been proven and there is no robust screening test.”

1.2.3 Incidence

Globally, the highest rates of iCCA are found in North East Thailand with 113 cases per 100,000 for men and 50 cases per 100,000 in women (Kirstein & Vogel, 2016). In Australia there are as few as 0.2 cases per 100,000 in men, and 0.1 per 100,000 in women, highlighting the significant geographical variation in cases (Tyson & El-Serag, 2011). In Thailand the incidence is linked to aetiology as the rates of liver fluke infections are endemic, as mentioned above.

The incidence of CCA also appears to vary based on the anatomical location of tumours. In the US the incidence of iCCAs have increased by 128% from 1973 – 2012 to 1.6 cases per 100,000 people, with mortality tripling in the decade leading up to 2008 (Kirstein & Vogel, 2016). In the UK there are 2.17 cases per 100,000.

Regrettably, as mentioned above, it is quite common for reported rates of disease to not differentiate between iCCA, pCCA and dCCA, making accurate estimates of their respective rates difficult to determine (Bridgewater et al., 2014). Tyson and El-Serag put it succinctly: “misclassifications can substantially impact the findings of epidemiological studies. Consequently, no definitive statement can be made on the temporal trends of cholangiocarcinoma in most world regions,” (Tyson & El-Serag, 2011).

1.2.4 Stage

The TNM classification is used to stage both iCCA and pCCA with a recent advance from the 8th edition of the AJCC, as can be seen for iCCA in figure 3, (Krasinskas, 2018).

Primary Tumor (pT)			
AJCC 7th Edition		AJCC 8th Edition	
pTX	Cannot be assessed	pTX	Primary tumor cannot be assessed
pT0	No evidence of primary tumor	pT0	No evidence of primary tumor
pTis	Carcinoma in situ (intraductal tumor)	pTis	Carcinoma in situ (intraductal tumor)
pT1	Solitary tumor without vascular invasion	pT1	Solitary tumor without vascular invasion, ≤5 cm or >5 cm
		pT1a	Solitary tumor ≤5 cm without vascular invasion
		pT1b	Solitary tumor >5 cm without vascular invasion
pT2	Solitary tumor with vascular invasion or multiple tumors, with or without vascular invasion	pT2	Solitary tumor with intrahepatic vascular invasion, or multiple tumors, with or without vascular invasion
pT2a	Solitary tumor with vascular invasion		
pT2b	Multiple tumors, with or without vascular invasion		
pT3	Tumor perforating the visceral peritoneum or involving the local extrahepatic structures by direct invasion	pT3	Tumor perforating the visceral peritoneum
pT4	Tumor with periductal invasion	pT4	Tumor involving local extrahepatic structures by direct invasion

Figure 3. Evolving iCCA staging classification.

From the 7th edition of the American Joint Committee on Cancer (AJCC) the TNM stage was incorporated and allows survival prediction and prognostic stratification (Banales et al., 2016). In both classifications there are 5 stages of detectable tumour, starting with the non-invasive tumour in situ (T_{is}), then the more recognisable stages T_{1-4}. The main differences between the 7th and 8th iteration are the introduction of size criteria for T1 tumours (without vascular invasion) and sub-categories to reflect this. There is also simplification of the T2 tumour stage, compared to the 7th edition.

From the SEER (Surveillance, Epidemiology and End Results Cancer Registries) database, a large cohort of 11710 patients with CCA were studied from 1973 to 2015. Most tumours were hilar (i.e., pCCA, 48.0%) closely followed by iCCA (46.6%) with only 5.3% being dCCA (Hang et al., 2019). Across all CCA, most tumours are stage IV (53.2%) followed by stages II (21.0%) and stage I (18.6%) with 6.6% being stage III and 0.5% stage 0 (Hang et al., 2019). Regrettably 49.7% of tumours (from 5611 patients) were so advanced that surgery was deemed inappropriate and therefore not undertaken. This is further evidence of the current unmet need for liver tumour patients as most present with advanced late-stage disease that are not amenable to curative treatment.

Given the previously reported issues with tumour location (iCCA vs. eCCA) another report has estimated that iCCA account for 6-10%, pCCA for 60% and dCCA 30% (A. S. Khan & Dageforde, 2019). For pCCA there is also the modified Bismuth-Corlette classification system for pCCA that considers the location of the stricture and contributes to other staging systems, but should not be considered a staging system in its own right (A. S. Khan & Dageforde, 2019).

1.2.5 Grade

Cholangiocarcinoma is usually graded as well, (~15-20%), moderately (~40-55%) or poorly differentiated (~30-40%) with similarity to normal cholangiocytes decreasing with worsening grade (Hang et al., 2019; Ma et al., 2019). Tumour grade is an independent prognostic predictor for both overall survival and cancer specific survival in iCCA (Ma et al., 2019). Poorly differentiated, high grade, tumours are also significantly associated with positive lymph node metastasis, another adverse clinical feature of iCCA (Martin et al., 2019).

1.2.6 Genetics of CCA

The genetics of CCA varies in sporadic versus infection associated cases with different mutational profiles reported with liver fluke (O. viverrini) related or unrelated CCA (Kongpetch et al., 2015). Viverrini-related CCA have mutations in TP53, KRAS, SMAD4, MLL3, RNF43, PEG3 and ROBO2 whereas epigenetic modulators such as BAP1, IDH1/2 and PBRM1 are more frequently mutated in non-viverrini-related CCAs (Kongpetch et al., 2015). However, TP53, ARID1A and KRAS mutations have also been shown in fluke-independent CCAs, demonstrating that molecular classifications may be of more relevance than aetiological cause, in this era of personalised medicine (Stavraka et al., 2019). So far, the role of immunotherapy in CCA has involved small

study numbers with modest responses to checkpoint inhibition in a selected cohort, and further studies are ongoing (Kelley et al., 2020).

Recurring evidence exists that drug-able pathways such as Ras/Raf/MEK/ERK, P38/MAPK, PI3K/mTOR pathways metabolic alterations (IDH1/2) as well as chromatin remodelling (ARID1A/ PBRM1/BAP1) have been identified by next generation sequencing to be involved in carcinogenesis (Xie et al., 2016).

Druggable molecular aberrations occur in iCCA with FGFR small molecule kinase inhibitors (FGFR2 fusions in 7-14% of iCCA), mutant IDH inhibitors (IDH1/2 mutants in 23-28%), HDAC inhibitors such given the BAP1 (9-12%) and ARID1A (15-36%) presence, MET kinase inhibitors (MET-HGF in 7%), Mcl-1 selective inhibitors (Mcl-1 in 16-21%), MEK inhibitors (KRAS (11-25%) and AKT/mTOR inhibitors (PI3K-AKT-mTOR in 4-8%) all being described (Rizvi & Gores, 2017). Identification of druggable targets such as these will help guide molecular sub-classification of CCA and is likely to increase in the future (Kirstein & Vogel, 2016). There is evidence that, in animal models, Beta-catenin and Wnt signalling drives CCA growth, with small molecule Wnt inhibitors reduces tumour growth and increases apoptosis (Boulter *et al.*, 2015).

1.2.6.1 UTAA in CCA

There have been a few studies of UTAA in CCA. Survivin has been reported in CCA previously in a series of 24 consecutive cases with 4 cases demonstrating strong nuclear protein with IHC which corresponded to a reduced overall survival (11 vs. 20 months, p= 0.033) (Javle et al., 2004). These results were not reproduced in a Chinese cohort of patients, where no correlations between Survivin levels and clinical features were found (Chang et al., 2004). Previous work combining both human CCA and animal models has found Survivin to be preferentially expressed in human tumour cells and also present in over 80% of tumour epithelial cells in a rat model of CCA (Boulter *et al.*,

2015). In addition to this, biliary excretion of Survivin has been used, in combination with serum CA 19-9, to help differentiate between benign and malignant biliary obstruction in human studies, indicating that Survivin may play a role in cholangiocarcinogenesis (Y. Liu et al., 2017).

Interestingly a carcinogenic toxin (Thioredoxin) secreted by liver fluke O. viverrini has been shown to up-regulate anti-apoptosis genes, including Survivin, in a cholangiocyte cell culture model (Matchimakul et al., 2015). In other cholangiocyte cell culture models, putative anti-tumour compounds (Guggulesterone and Curcumin) successfully induced caspase-dependent apoptosis and downregulated Survivin levels, as detected by Western Blot (Koprowski et al., 2015; Zhong et al., 2015).

Telomerase protein has been detected in iCCA and pre-neoplastic lesions, using in situ hybridisation and in a separate study, telomerase activity has been detected in CCA biopsy samples (using the TRAP assay) indicating its potential role in CCA (Itoi et al., 2000; Ozaki et al., 1999). Furthermore, quantitative PCR has also detected hTERT mRNA in both HCC and biliary tumour tissues whilst noting its absence in background tissue and GIST tumours (Udomchaiprasertkul et al., 2008).

Serum hTERT mRNA has been detected in 85% of CCA patients, and is absent in healthy volunteers, indicating a potential role as a biomarker (Leelawat et al., 2006). However, more recent research assessing the expression of hTERT mRNA in cell lines and primary human hepatocytes (PHH) has found that Telomerase is present in cholangiocytes but at significantly lower levels than PHHs (Qin et al., 2018). These findings vary between cell lines, so should be interpreted with caution (Lie-A-Ling et al., 2006; Qin et al., 2018). A recently developed telomerase inhibitor, KML001, is well tolerated in advanced CCA patients but has failed to demonstrate anticancer effects (Jo et al., 2019).

1.2.7 Prognosis

Complete surgical resection currently the only curative treatment option in CCA (Ting et al., 2016). Whilst chemotherapy, loco-regional therapies or radiotherapy can prolong survival, patients with more advanced disease are precluded from surgical management (Raoof et al., 2017). Comprehensive assessment of the extent of tumour disease, by cross sectional imaging, as well as ensuring adequate liver function are necessary to ensure operations are undertaken on appropriate patients (A. S. Khan & Dageforde, 2019). Adverse features include tumour number, tumour size, vascular invasion, lymph node metastasis, advanced tumours stage at diagnosis, metastatic disease, poor tumour grade, male gender and increased age (Ma et al., 2019; Raoof et al., 2017; Wang et al., 2013).

The presence of adverse clinical features (multifocal disease, large vessel vascular invasion and/or metastatic spread) determine whether or not a CCA is technically-resectable. Median survival in CCA is 24 months, but a dismal 3.9 months in untreated, unresectable disease (Krasinskas, 2018). In patients with unresectable CCA given chemotherapy, overall survival increases to 8.1 months with Gemcitabine and is further lengthened with the addition of Cisplatin to 11.7 months. Five-year survival rates for surgically resected tumours ranges from 25 – 50% with poorer outcomes in patients with positive surgical margins giving a median survival of 12-21 months in these cases (A. S. Khan & Dageforde, 2019).

1.2.8 Treatment

This section will review the treatment options for iCCA only.

1.2.8.1 Surgical

Surgery is the only curative option but requires that sufficient functional liver will remain post-procedure and that the disease is not late stage (A. S. Khan & Dageforde,

2019). Around 12-40% of iCCA are surgically resectable at presentation, but this requires a highly technical and extensive operation (Mazzaferro et al., 2020). Nearly 80% of resectable iCCA require an extended hepatectomy and 29% of cases undergo extra-hepatic bile duct resection (Bridgewater et al., 2014). A recent meta-analysis has found that down-staging tumours (with chemotherapy, radiotherapy or TACE) prior to resection significantly improves overall survival from 12 to 29 months, $P< 0.001$ (Kamarajah et al., 2020). Tumours with pathologically or clinically positive lymph nodes benefit from chemotherapy post resection. In clinically positive LN metastatic disease, combining surgery and chemotherapy significantly improves survival compared to surgery alone (+10.1 months) or chemotherapy alone (+10.6 months) indicating the benefit of a combined approach (Martin et al., 2020).

Liver transplantation for iCCA remains controversial. In highly selected cohorts, liver transplantation may be of benefit and is not currently offered to patients, but this remains a contentious issue. Intrahepatic tumours <2cm (very-early iCCA) have a 5-year recurrence rate of 18%, compared to 61% for multifocal tumours, or tumours >2cm in size (Vogel & Saborowski, 2017). In transplantation for iCCA, irrespective of the tumour size, >50% of patients have recurrence within 2 years with 2-year survival (48%) and 5-year survival (23%) reflecting an aggressively recurrent disease (Mosconi et al., 2009). In the rare cases of very-early iCCA in patients that are not resection candidates (e.g., due to cirrhosis) a multicentric single arm clinical trial is currently under way to confirm the effectiveness of liver transplantation (Mazzaferro et al., 2020). In the more commonplace cases with iCCA >2cm with no evidence of vascular spread, or extrahepatic disease, neoadjuvant chemotherapy has been used as a bridging treatment to transplant, and recommenced 4-6 weeks post-transplant, achieving an impressive 5-year overall survival rate of 83% (Lunsford et al., 2018). This is further

evidence that liver transplantation may become a standard treatment in selective cases of iCCA.

1.2.8.2 Locoregional Therapies

Locoregional treatments can be used with life prolonging intent or to achieve tumour downstaging to facilitate surgery. In stage III disease, confined within the liver, locoregional therapies (RFA/TACE/TARE) can be used to treat patients with palliative intent (Bridgewater et al., 2014). In lymph node positive iCCA, there is no survival benefit of surgery compared to TACE, with fewer adverse outcomes in the non-operated cohort (Scheuermann et al., 2013). However, adverse clinical measures such as a highly vascular tumour, or Child Pugh B cirrhosis are poor prognostic features when TACE is used, highlighting the importance of judicious patient selection for medical therapies (Vogl et al., 2012). Intrahepatic CCA are not usually hypervascular (unlike most HCCs) but can demonstrate some degree of vascularity on angiography, further complicating whether or not these tumours are amenable to TACE (Bridgewater et al., 2014). Convincing evidence for the role of neoadjuvant TACE and TARE in iCCA is currently lacking and requires further research (Massironi et al., 2020; Mazzaferro et al., 2020). Other down staging techniques are available, such as pre-surgical selective internal radiation therapy (SIRT) in conjunction with chemotherapy in iCCA in achieving an 18% rate of conversion to R0 resection with no significant toxicity (Mazzaferro et al., 2020).

1.2.8.3 Systemic Therapies

Combination chemotherapy (Gemcitabine & Cisplatin) improves overall survival (compared to Gemcitabine alone) from 8.1 months to 11.7 months when there is extrahepatic disease (A. S. Khan & Dageforde, 2019; Valle et al., 2010). Another combination therapy is Gemcitabine and Oxaliplatin which increases overall survival to 12.4 months, with fewer side effects that may be better tolerated by patients (Massironi

et al., 2020). There is also increasing evidence that combination therapies give better survival statistics, regardless of whether it is dual-drug treatment, or a combination of chemo-radiotherapy (Bridgewater et al., 2014).

Promising targets for personalised medicine in iCCA include FGFR small molecule kinase inhibitors, IDH mutant inhibitors and checkpoint inhibitors (PD-1) that are currently under investigation as potential treatment options (Massironi et al., 2020; Rizvi & Gores, 2017). Checkpoint inhibition may be clinically useful in selected cases of CCA, with further results expected imminently, (Kelley et al., 2020).

1.3 Mixed Hepatocellular-Cholangiocellular Tumours

Mixed Hepatocellular-Cholangiocellular tumours are a rare form of PLC, comprising around 1% of all liver tumours (Moeini et al., 2017). By their very nature, expressing characteristics of both CCA and HCC, mixed (sometimes called combined) liver tumours are a diverse group displaying varying similarities to HCCs and CCAs. When comparing mixed tumours with HCCs and CCAs, mixed tumours display intermediate overall survival and prognostic behaviours, as they are sandwiched between the poorer prognosis of CCA and the relatively better prognosis of HCC (A. Q. Wang et al., 2016). Mixed tumours have traditionally been graded like CCAs and have a poorer prognosis than HCCs (Joseph et al., 2019).

However, at the genetic level, mixed tumours are more akin to HCCs despite some similarities with CCAs being present. The tumour suppressor TP53 and ARID1A are commonly altered in both CCA and mixed tumours but $pTERT^{Muts}$, BRAF and FGFR2-BICC1 alterations are specific to mixed tumours (Moeini et al., 2017).

The polymorphous nature of mixed tumours makes classification somewhat difficult, however the WHO recognises both the classical and the less common stem cell phenotypes of mixed HCC-CCA (Stavraka et al., 2019). A significantly rarer tumour

type called a collision tumour is found to have both CCA and HCC occurring separately with no transition region from one cellular morphology to another (Stavraka et al., 2019).

1.3.1 Aetiology

There is currently no international consensus on the aetiological causes of mixed PLC (Stavraka et al., 2019). The most recent WHO classification has placed all PLCs on a spectrum, with HCC and CCA at the extremes and mixed tumours, with all their inherent heterogeneity, somewhere in between (Sciarra et al., 2020). Most mixed tumours occur in the context of cirrhosis with viral hepatitis, autoimmune diseases, fatty liver disease, alcohol consumption and congenital cystic diseases of the hepatobiliary tree also playing a significant role (Mok et al., 2016).

1.3.2 Incidence

The demographic profile of mixed tumours is incomplete because of its rarity (Stavraka et al., 2019). It is known that mixed tumours account for 1.1% of PLCs (53 of 5049) with three tumour subtypes (Yoon et al., 2016). Type A/type I mixed tumours (also called collision tumours) are when simultaneous HCC and CCA are coincidentally detected, type B/II are when the tumour is truly transitional with elements of both HCC and CCA intermingled with type III being a fibrolamellar HCC with mucin producing glands within the same tumour (Jarnagin et al., 2002). Allen & Lisa are responsible for the alphabetical (and excluded FL-HCC appearing tumours from their initial analysis) and Goodman et al for the numerical nomenclature (Allen & Lisa, 1949; Goodman et al., 1985).

Mixed tumours appear to occur in approximately equal measure across both sexes (52% M, 48% F) and share demographic and clinical features of CCA (Jarnagin et al., 2002). When previously there was increasing data that mixed tumours should be considered as

distinct entities from both CCA and HCC, and not a subclass of each respective parent (Wachtel et al., 2008). But genomic profiling of mixed tumours has highlighted more similarities to HCCs with pTERT^{Muts} and TP53 both occurring in 80% of cases (Joseph et al., 2019). Clearly mixed tumours are highly complex in their nature and are not yet fully described.

1.3.3 Genetics

As mixed tumours possess morphological features of both CCA and HCC, it would be prudent to assume the genetic profiles also reflect this. Gene expression profiling, DNA copy number and exome sequencing of another cohort of mixed tumours highlight classical-type mixed tumours express genetic components of both HCC and iCCA whilst iCCA and HCCs are completely distinct entities (Moeini et al., 2017). Mixed tumours with stem cell features are more HCC-like, but still possess some characteristics of iCCA. Indeed, current diagnostic advice highlights the high degree of heterogeneity in morphology, immunophenotype, molecular biology and clinical features mixed tumours possess, making diagnosis difficult (Sciarra et al., 2020).

Given the clinical course and prognosis of mixed CCA-HCC is more similar to CCA with staging and treatment algorithms reflecting this, it is surprising to learn the most recent evidence actually indicates to opposite. With pTERT^{Muts}, and TP53 alterations in 80% of mixed tumours (in common with HCCs) but no IDH1/2, FGFR2 or BAP1 (similar to iCCAs) found with next generation sequencing technologies, it appears mixed tumours are more similar to HCC than previously thought (Joseph et al., 2019). However, these findings are somewhat uncertain as other researchers have used online compendiums of genetic data (*GeneCards*) and found that mixed tumours are predominantly a subtype of CCA (Mok et al., 2016). Some genetic commonality does exist between HCC, iCCA and mixed tumours, such as ARID1A and TP53 alterations but most differences are more nuanced, as shown in table 1 (Stavraka et al., 2019).

HCC	CC	cHCC-CC
Loss of heterozygosity at chromosomes 8 p, 17q, 4q, 16q, 13q, 6q, and 7 p	Microsatellite instability (MSI-H)	Loss of heterozygosity at chromosomes 3q, 14q
Inactivation of tumor-suppressor genes such as TP53	TP53 mutations	TP53 mutations
Activation of oncogenes such as CTNNB1/beta-catenin and Wnt pathway	K-RAS mutations	Rb-1 locus replication error pattern
ARID1A	ARID1A BPRM1 IDH1/IDH2	ARID1A CTNNB1 TP53 RYR3 FBN2
		MYC amplification

Table 1. Genetic alterations in hepatocellular carcinoma (HCC), cholangiocellular carcinoma (CC) and combined HCC-CCA (cHCC-CC).

1.4 Universal Tumour Associated Antigens

The Universal Tumour Associated Antigen (UTAA) describes the presence of proteins found widely expressed in malignancies (Gordan & Vonderheide, 2002; Paschen, 2009). UTAA are a diverse group of proteins that are rarely found in healthy adult homeostasis yet appear to flourish in, and are detectable across, a wide range of solid organ and haematological malignancies (Altieri, 2008). Similar mechanisms are deployed for the evasion of normal homeostatic control by a wide range of malignant neoplasms and the antigens associated with them have been detected across a variety of tumour types. This has lead to UTAA being described as possessing the *Hallmarks of Cancer* (Hanahan & Weinberg, 2000, 2011). Resisting cell death and enabling replicative immortality are two of the six proposed mechanisms that are required for cancerous cells to evade homeostatic control (Hanahan & Weinberg, 2000, 2011) with the ability of cancerous cells to inhibit apoptosis (Survivin) or to enable replicative immortality by increasing telomere length (Telomerase) being archetypal methods by which tumours achieve these *Hallmarks of Cancer*.

There has been some characterisation of Survivin, in cancers of the lung (Y. Xie et al., 2012), brain (Fangusaro et al., 2005), cervix (Zannoni et al., 2014) and breast (Stoetzer et al., 2013) where it has been associated with poorer prognosis, resistance to current

therapies and higher recurrence rates (Altieri, 2008). There is ongoing work to describe the exact mechanisms by which Survivin has these effects in tumour biology.

The rate-determining enzyme for telomere extension, Telomerase, has been reported to be present in up to 98% of cancer cell lines and 90% of 101 biopsies from twelve tumour types (Kim et al., 1994). Telomerase catalyses the addition of the hexameric repeat (TTAGGG) to the ends of telomeres thus reversing the natural shortening that telomeres undergo during cell division. This lengthening of telomeres increases the theoretical Hayflick limit that ultimately results in cellular senescence after a finite number of cell divisions.

1.5 Survivin

Survivin was first described in 1997 in malignantly transformed cell lines as well as being found in foetal tissues (Ambrosini et al., 1997). Survivin has also been reported as being the fourth most commonly transcribed protein that is elevated in human cancers, indicating its clinical relevance across a number of tumour subtypes (Velculescu et al., 1999).

Survivin is a member of the inhibitor of apoptosis (IAP) family of eight genes and, at 142 amino acids in length (16.5kDa) is the smallest. It has a number of functions within the cell (Wheatley & Altieri, 2019) and is characterised by the presence of a single copy of the Baculovirus IAP Repeat (BIR) that is required for apoptosis inhibition (Athanasoula et al., 2014). There is incomplete understanding of the roles undertaken by Survivin in various cellular compartments and processes, and it appears that this curious ‘adaptor protein’ is able to interact with, and transport, other proteins to their required destination (Wheatley & Altieri, 2019).

1.5.1 Historical information & discovery

Survivin was first described in foetal tissues, solid organ tumours and haematological malignancies in the late 1990s (Ambrosini et al., 1997). With undetectable levels of Survivin mRNA in terminally differentiated adult tissues (with the exception of thymus and to a lesser extent, placenta and testes) and a re-appearance in adult malignancies, Survivin has courted interest as a potential biomarker and therapeutic target for more than 20 years. More recent work has also found Survivin in components of the immune system, such as B and T lymphocytes, and occasionally in cells with a high turnover rate, such as the basal keratinocytes of the skin as well as the cells of the gastrointestinal tract (Dallaglio et al., 2014; Lorenzetti et al., 2019).

1.5.2 Structure & Function

Survivin is vital for physiological cell division as homozygous deletion is incompatible with embryonic development (Altieri, 2010). The structure of the 142 amino acids is integral to Survivin's numerous functions. With an N-terminal baculovirus inhibitor of apoptosis repeat (BIR, aa 20 - 90) containing a zinc-binding domain, linked to a C-terminal alpha helix (aa 98 – 142) that facilitates homo-dimerisation of the Survivin monomer, this small protein has a complex structure, as determined by X-Ray crystallography (Noel et al., 2000; Wheatley & Altieri, 2019). Alongside this alpha helix, three other proteins can associate and create the chromosomal passenger complex (CPC) whose presence is essential for mitosis to occur.

In normal cell division, the constituent parts of the CPC comprise three proteins (Survivin, Borealin and the inner centromere protein: INCEPT) and an enzyme (Aurora B kinase) which are essential for chromosome condensation, spindle assembly, ensuring microtubule-kinetochore alignment and cytokinesis (Jeyaprakash et al., 2007). A tri-helical bundle forms from each of the three non-enzymatic component parts and in

addition to Aurora B kinase forms a novel enzymatic subunit whose exact functions are difficult to fully assess, given the interdependence of the constituent parts, but are nonetheless vital for their functionality (Jeyaprakash et al., 2007).

Survivin is involved in a positive feedback loop by utilising the alpha helical binding region to detect phosphorylated histone during mitosis (as a part of the CPC). This recruits chromatin whilst activating Aurora B which in turn phosphorylates Survivin, resulting in activation and ensuring chromosomal orientation during mitosis (Kelly et al., 2010; Wheatley & Altieri, 2019). The CPC localises to the chromosomes, then the inner centromere during mitosis and is intricately involved in the orchestrated movement of the microtubules, mitotic spindle assembly and cytokinesis required for cellular division (Athanasoula et al., 2014; Rosa et al., 2006). Maximal expression of the CPC occurs in the G2/M phase of the cell cycle with interruption of this association resulting in a loss of apoptosis inhibition (F. Li et al., 1998).

The exact location of Survivin during mitosis is somewhat contentious as there is evidence that demonstrates both tubulin (microtubule and centrosome) association and Survivin residing within in the CPC, both of which are detectable with novel tracer-technologies that can track Survivin movements in real time and confirmed their likely presence in these distinct subcellular pools (Beghein et al., 2016). These distinct subcellular pools are felt to play differing roles throughout the cell cycle and remain incompletely understood. Expression of Survivin has been demonstrated in peroxisomes (Beghein et al., 2016), mitochondrial (Dohi et al., 2004), cytoplasm (Shimamoto et al., 2009), and the nucleus (Dallaglio et al., 2014).

Apoptotic pathways are classified as intrinsic or extrinsic depending on the mechanisms involved. Extrinsic pathways are triggered by ligation of the ‘death receptors’ of the Tumour Necrosis Factor-Receptor (TNF-R) family, whilst intrinsic pathways are triggered by stimuli (such as irradiation or cytotoxic drugs) that leads to cytochrome-C

release from mitochondria, ultimately triggering caspase-9 activation (Athanasoula et al., 2014). Survivin is unable to interact with caspases by itself but can interact with XIAP or hepatitis B virus X-interacting protein (HBXIP) to inhibit caspase activation and thus reduce apoptosis (Peery et al., 2017; Wheatley & Altieri, 2019). The Survivin-XIAP complex stabilises XIAP and increase their anti-caspase potency, whilst sequestering XIAP from the pro-apoptotic smac/DIABLO, which remains within the mitochondria which is itself retained by intra-mitochondrial Survivin (Athanasoula et al., 2014).

The role of Survivin in cancer centres on the loss of normal, cell cycle dependent, expression. De-repression of the naturally cyclic Survivin expression results in continuous synthesis throughout the cell cycle (Wheatley & Altieri, 2019). This results in the unrestrained expression of Survivin in cancerous tissues. There are mentions in the literature of sometimes contradictory findings when classifying subcellular localisation in tumours when comparing survival statistics, or disease stage. Specifically: cytoplasmic positive staining in CRC being associated with more advanced disease stage and poorer overall survival, (Shimamoto et al., 2009). This is contradicted by correlations between nuclear positivity of both premalignant and malignant skin lesions (highest expression in poorly differentiated squamous cell carcinoma) when compared to normal skin (Dallaglio et al., 2014).

Yet more complexity, and confusion, arises when considering that some published works have not differentiated between subcellular locations of positive staining as peroxisomal and mitochondrial localisation will be indistinguishable from cytoplasmic pools of Survivin, unless specifically searched for (Wheatley & Altieri, 2019). Needless to say, there is much scope for expanding these fields of study to potentially clarify the independent roles played by Survivin during cellular respiration, and how these are exploited in both pre-cancerous and cancerous growths.

1.5.3 Isoforms & Location

Six isoforms of Survivin have been described with varying degrees of understanding as to their exact cellular function, with a further 4 (as yet undetected) hypothetical isoforms (Sah & Seniya, 2015). Wild type, DeltaEx3, 3alpha and 3B appear to have anti-apoptotic actions, whilst 2B has reduced anti-apoptotic functionality owing to a truncated functional (BIR) domain and 2Alpha have apparent pro-apoptotic properties (Mishra et al., 2015; Sah & Seniya, 2015). However, some conflicting results have emerged as to the precise interactions between the variants, with a theorised role for heterodimers between WT Survivin and the 2alpha or 2B isoforms, being inconsistently demonstrated by various research groups and isoforms 2alpha, 2B and 3B apparently not interfering with cellular division in any capacity (Athanasoula et al., 2014). There is clearly potential for more work to be done to elucidate the mechanisms involved in these processes.

Wild type Survivin associates with microtubules of the mitotic spindle in the G2/M phase of the cell cycle that results in a breech in the apoptotic checkpoint, where aberrant cells would normally proceed to programmed cell death (F. Li et al., 1998). The subcellular location of Survivin was thought to be either microtubule associated or as a chromosomal passenger complex, however in HeLa cells roughly 80% of Survivin is cytosolic, with the remaining 20% being nuclear (Fortugno et al., 2002).

Various authors have described poor outcomes when nuclear Survivin has been detected. Hasby & Mokhtar found that 90% of HCCs (n=20) expressed nuclear Survivin in a small Egyptian cohort of patients, with an increased expression in poorer grades of tumour, and less prevalent in cirrhotic control samples, when measuring IHC staining intensity (Hasby & Mokhtar, 2010). In a larger cohort of German patients with oesophageal squamous cell carcinoma (n = 84), nuclear expression was detected in 80 % (n = 67) and associated with shorter overall survival (p= 0.003) whereas cytoplasmic

Survivin was noted in 63% of cases (n = 53) and had no prognostic relevance (Grabowski et al., 2003). Similarly, when studying colorectal cancer, Nuclear Survivin was correlated with metastatic disease (p = 0.026) whilst cytoplasmic staining did not correspond to any disease parameter (Jakubowska et al., 2016).

In non-small cell lung cancer, there has been sufficiently contradictory evidence on the prognostic relevance of nuclear Survivin that a meta-analysis was undertaken to assess the overall results (Y. Xie et al., 2012). Eight hundred and twenty-three patients were included in the pooled analysis and IHC was used to assess the location of Survivin in the tumour tissues. When the results were viewed overall, there was no significant risk of having nuclear Survivin staining (HR = 1.54, 0.79 – 3.02) but when taking into account the ethnicity of the study participants, nuclear Survivin was associated with reduced survival in Caucasians with NSCLC, HR = 2.38 (1.60 – 3.43). The authors conclude that nuclear Survivin is associated with poor prognosis in Caucasians with NSCLC, which introduces the potential for a potential genetic, or even geographical variation in Survivin expression rates.

However, cytoplasmic Survivin has been reported in 69/71 (97.2%) of cases of locally advanced cervical cancer with a mere 7 cases of nuclear staining in the same cohort (9.8%) (Zannoni et al., 2014). Women with high cytoplasmic levels had a shorter disease-free survival (5-year DFS 80.8% vs 55.3%, p = 0.033), a positive lymph node status (p = 0.036) and residual tumour (p = 0.016) with no correlation between nuclear expression of Survivin and disease characteristics.

It is apparent from the literature that Survivin is detected in a wide range of tumours, and in some pre-malignant lesions, with varying implications for tumour stage, grade, or size as well as prognosis.

1.5.4 Pathophysiology

As described above, accumulations of Survivin have traditionally been described as either nuclear or cytoplasmic in nature, but the real location of these fractions is more nuanced. As mentioned above: nuclear, cytoplasmic, mitochondrial and peroxisomal stores have been described previously (Athanasoula et al., 2014; Beghein et al., 2016). The role of inter-compartmental shuttling of Survivin has been proposed as a potentially key step in carcinogenesis. Nuclear Survivin is transported to the cytoplasm because of a nuclear export signal (NES) with in a leucine-rich region of the molecule, and is exported by the evolutionarily conserved export receptor Crm1 (Knauer et al., 2007). NES-region knock out studies and NES-specific antibodies have abrogated Survivin's ability to be exported from the nucleus and inhibit apoptosis, which was confirmed in a small IHC-based cohort of CRC patients, who had poorer prognoses with cytoplasmic Survivin (Knauer et al., 2007). However, these findings have not been replicated in other tumour types, with a plethora of tumours correlating a worse prognosis with nuclear expression of Survivin. The importance of intermolecular interactions when the CPC is formed is highlighted by the findings that both deltaEx3 and 2B isoforms have a reduced affinity for Borealin and hence do not locate to the CPC (Noton et al., 2006).

The relevance of Survivin's interactions during physiological and pathological processes become apparent when targeted therapies are considered. The broad categories include the inhibition of partner-protein interactions, homodimerisation, gene transcription, as well as inducing degradation of Survivin mRNA, or exploiting Survivin's peptide sequence by using a vaccine-based immunotherapeutic approach (F. Li et al., 2019).

1.5.4.1 Detection/ Promoter variants

In 2004, Xu and colleagues sequenced cancerous cell lines and compared them to normal counterparts noting a few single nucleotide polymorphisms (SNPs) present in

the promoter region of the BIRC5 gene encoding Survivin (Y. Xu et al., 2004). The most intriguing of these SNPs (31 base pairs upstream of the transcription start site, position -31, C to G 'mutant') results in increased expression of both Survivin mRNA and protein and has subsequently been assigned the unique positional code *rs9904341*. As had previously been noted, there are several CDE/CHR transcriptionally sensitive regulatory repressor domains that control transcription in the G1 phase of the cell cycle. These can be found in the promoter region of Survivin (CDE: -6, -12, -171. CHR: -42) carrying the sequence motifs GGCGG and ATTTGAA respectively (F. Li et al., 1998). Mutations in these repressor domains would result is de-repression of Survivin in the G1 phase resulting in increased transcription and hence, higher levels of both mRNA and protein.

1.5.4.2 Protein Expression & Detection

Commercially available enzyme linked immunosorbent assay (ELISA) kits have been used to detect Survivin in peripheral blood samples specimens in a variety of cancers. There has been reported success in correlating serum Survivin in pancreatic cancer that correlated with disease parameters such as perineural invasion, venous invasion, lymph node spread metastasis, cell differentiation and recurrence (Dong et al., 2015). Serum Survivin was also found to be higher in patients with malignant (compared to benign) brain tumours, and also associated with the GG promoter at the -31 position (Kafadar et al., 2018). The relationship between a hepatobiliary tumour subtype (Gall bladder carcinoma) has been correlated with higher Survivin levels with adverse clinicopathological features such as advanced stage of disease and moderate/poor differentiation of tumour cells (Nigam et al., 2014).

However, these findings need to be taken in context of other reports claiming that serum Survivin is not useful in the identification of patients with cancer. Jakubowska and colleagues reported higher serum Survivin in healthy controls compared to patients with

colorectal cancer (81.8% compared to 38.2%), albeit in a small single centre study of 55 patients with CRC with 22 control subjects (Jakubowska et al., 2016). Jia and co-workers have stated unequivocally similar views in their paper titled '*Survivin is not a promising serological maker for the diagnosis of hepatocellular carcinoma*' (Jia et al., 2015). This somewhat damning statement is backed by evidence from two separate commercially available ELISA kits (R&D and Abnova) that found no significant difference when measured in HCC patients and healthy controls, and also found an extremely low correlation coefficient (0.0064, p=0.481) when comparing identical serum samples with the different kits (Jia et al., 2015).

1.5.4.3 Global Variation

When Turkish brain tumours were assessed for the -31C/G promoter variant (*rs9904341*) and compared with serum levels of Survivin, significantly higher levels were detected in the GG homozygotes (Kafadar et al., 2018). However, *rs9904341* GG homozygous breast cancer patients in Egypt had a better prognosis and lower levels of serum Survivin, indicating some potential differences between tumour type or geographic variation (Motawi et al., 2019). In a case-control study of Serbian paediatric Wilm's Tumours found GG homozygotes were at a higher risk of developing cancer, and those with cancer and the GG variant had a poorer prognosis (Radojevic-Skodric et al., 2012).

However, a recent meta-analysis of case-control studies across a broad range of cancers has found Asian *rs9904341* CC homozygotes are significantly more likely to develop cancer, with no equivalently significant relationship in Caucasians (Moazeni-Roodi et al., 2019). More work describing potential ethical variations is required, especially as no studies have been undertaken in the British population thus far.

1.6 Telomerase

Telomerase is the enzyme required for telomere extension, and is conserved across many eukaryotic species (Wu et al., 2017). The enzyme facilitates the lengthening of telomeres, thereby increasing a cells ability to continue dividing beyond the physiologically pre-determined number of cell divisions. Eukaryotic DNA polymerase exploits an RNA primer when initiating DNA synthesis in the 5' to 3' direction and when it is later removed, this leaves up to 200 nucleotides uncopied (Singh et al., 2015). The aptly named *end replication problem* describes these missing 100-200 base pairs per cell division (Musgrove et al., 2018). Constant shortening of linear chromosomal ends during each cellular division ultimately results in cell cycle arrest, senescence or apoptosis (Singh et al., 2015). Therefore, a mechanism to halt or reverse this division-shortening effect has been shown to increase the number of cellular divisions, and immortalising the cell.

Telomerase has been detected in germline cells, haematopoietic cells, stem cells as well as in rapidly renewing cells and in cells with a high mitotic rate (Leão et al., 2018). Initial work described the expression of Telomerase in predominantly embryonic and adult germline tissues, such as foetal and adult Testes and ovaries, and their absence in oocytes and sperm (Wright et al., 1996). In normal human cells Telomerase remains unexpressed, however it has been quoted as having detectable levels of activity in 98/100 immortal cell lines as well as in 90/101 human cancers (Kim et al., 1994).

1.6.1 History & Discovery

Early work describing the presence of Telomerase in yeast, *Tetrahymena*, by Greider & Blackburn was undertaken in 1985 (Greider & Blackburn, 1985). This work has more recently earned them the 2009 Nobel Prize in Physiology/Medicine, an honour they shared with Szostack for the simultaneous nature of their work on the enzyme (Szostak

& Blackburn, 1982). The initial findings demonstrated Telomerase to be a ribonucleoprotein that required the presence of both the catalytic protein, encoded by hTERT, as well as the RNA subunit, hTERC or hTR, to be functionally active (Shay & Wright, 2019). Over the decades since the breakthrough discovery of Telomerase it has been found to be conserved across as a wide range of species and the structure in humans was finally described in 2018 using cryo-electron microscopy (Nguyen et al., 2018). Whilst the sequences of the catalytic component of Telomerase (TERT) are conserved over many species, there is considerable divergence in the sequences of integral Telomerase-RNA (TR/ TERC) across species (Musgrove et al., 2018; Nguyen et al., 2019). These differences highlight the fact that telomerase physiology is incompletely understood. However, there is evidence that the human TR/TERC (hTERC) is expressed in a wide range of tissue types whilst the catalytic subunit (hTERT) is only present in the malignant or premalignant state (Leão et al., 2018). This further emphasises importance of understanding the role played by hTERT in human tumours, and whether it can be used as a potential biomarker. As hTERT is widely expressed across a range of neoplastic growths, any findings in liver tumours may have implications beyond this one organ.

Telomerase facilitates the hexameric repeat (TTAGGG) being added to the ends of telomeres, thus reversing the natural shortening that telomeres undergo during cell division. As approximately 100 - 200 base pairs are lost from chromosomal end-sequences with every cellular division (Singh et al., 2015). Telomere extension increases the theoretical Hayflick limit thus delaying the ultimate state of cellular senescence by extending the finite number of cell divisions that can occur (Shay & Wright, 2011). Hence the cellular clock is being reset, allowing cells to develop replicative immortality.

1.6.2 Structure

Structural studies of human Telomerase have been challenging due to its very low abundance (Nguyen et al., 2019). The telomerase ribonucleoprotein includes the catalytic telomerase reverse transcriptase (TERT) and telomerase RNA component (TR/TERC) that includes the internal template required to extend telomeres (Musgrove et al., 2018). The ribonucleoprotein complex has a characteristic called *repeat addition processivity* (RAP) that allows translocation of and realignment to the RNA template to allow multiple TTAGGG repeats to be added to the 3' chromosomal end, the specifics of which are still under study (Musgrove et al., 2018). Various other proteins interact with the TERT-TERC complex, in humans these are called small Cajal body ribonucleoprotein (scaRNP) which includes dyskerin, NOP10, NHP2 and possibly GAR1 (Chan et al., 2017).

The Telomeric DNA complex, and support machinery includes the Shelterin complex (Leão et al., 2018). The Shelterin complex includes TRF1, TRF2, POT1, TIN2, RAP1 and TPP1 and is able to locate Telomeres by TTAGGG recognition motifs in TRF1, TRF2 and POT1 (Singh et al., 2015). TRFs 1 and 2 bind double-stranded DNA, POT1 binds ssDNA and TIN2 and TPP1 mediate shelterin assembly and TERT recruitment (Chan et al., 2017). Hence, recruitment of TERT-TERC complexes to the chromosomal ends is due to the presence of the Shelterin complex of proteins and is stabilised by dyskerin (Chan et al., 2017; Nault et al., 2019).

1.6.3 Pathophysiology

The pre-determined number of cellular divisions that are possible in terminally differentiated somatic cells is due to the length of telomeres. There is a shortening of between 100 - 200 base pairs during each cellular division because of the removal of the RNA primer/template from the DNA template, leaving an uncopied single strand of

DNA exposed and uncopied into the daughter cell (Singh et al., 2015). As Telomerase is able to extend telomeres a new equilibrium is reached in malignant cells with a balance between telomere extension and degradation being achieved and therefore extending the previously finite number of cellular divisions into the realms of the infinite.

1.6.3.1 Telomerase Promoter Mutants

The promoter region of a gene is upstream of the 5' transcription start site and facilitates the binding of RNA polymerase, alongside other transcription factors that allows transcription to initiate. Mutations in the promoter regions ($pTERT^{Muts}$) can disrupt normal cellular processes and can result in an increase or decrease of mRNA, and subsequent protein expression (de Vooght et al., 2009).

Whilst $pTERT^{Muts}$ were initially found in familial and sporadic malignant melanoma there have been cases reported in a wide range of cancers, including approximately 60% of hepatocellular carcinomas (Nault et al., 2013) making it the most common genetic aberration in HCCs, (Zucman-Rossi et al., 2015). The mutations in question are -124/C228T and -146/C250T, are mutually exclusive, and are associated with increased Telomerase expression and activation in other tumour types such as glioblastoma, urothelial carcinoma, oligodendroglioma, medulloblastoma and thyroid carcinoma (Huang et al., 2015).

Mutations in the promoter region of the hTERT gene (-146/C250T and -124/C228T) creates a new consensus motif that facilitates transcription machinery binding to the promoter region of the gene (Akincilar et al., 2016). The E-Twenty-Six (ETS) family of transcription binding motifs has 27 members, with a specific member (GABPA) demonstrating C250T and C228T specific binding and increases in transcription of Telomerase (Akincilar et al., 2016). These $pTERT^{Muts}$ are heterozygous and occur in transcriptionally active, unfolded chromatin (Stern et al., 2015). Clinically, $pTERT^{Muts}$

are highly correlated with increased transcription as well as increased Telomerase activity (Huang et al., 2015).

Telomerase promoter mutations occur in a wide range of tumour types, stages and grades and are thought to be an early driver mutation for tumour development (Leão et al., 2018). The presence of these mutations in pre-neoplastic nodules of cirrhotic patients, as well during the metaplastic processes Hepatic Adenomas undergo to progress to HCC, confirms that this can be an early step in tumourigenesis in liver tissues (Nault et al., 2013). Also, $pTERT^{Muts}$ occur with increasing frequency from low grade though to high grade dysplastic nodules as well as in early HCCs, further evidencing the early-carcinogenic nature of this common genetic alteration (Nault & Zucman-Rossi, 2016). Intriguingly the prevalence of $pTERT^{Muts}$ in HCCs varies with geographic region, with more mutants in Western populations (around 60%) compared to those studied in the East (~30%) (Huang et al., 2015; Schulze et al., 2016).

Techniques used to identify $pTERT^{Muts}$ include Sanger sequencing PCR products from fresh frozen tumour tissues (Nault et al., 2013), next generation sequencing of frozen tumours (Nault et al., 2017) as well as sequencing DNA extracted from formalin fixed paraffin embedded (FFPE) tissues (Huang et al., 2015). Analysis of the archived (FFPE) tissues has used amplification of specific sequences with polymerase chain reaction (PCR) and either analysis by restriction fragment length polymorphism (RFLP) or, less frequently, Sanger sequencing. Notably the majority of small-scale studies have used the PCR-RFLP approach rather than more modern techniques.

1.6.3.2 Other Genomic Alterations

Telomerase expression is under the control of genes that are known to be highly expressed in the liver (Nault et al., 2019). Viral insertion into hepatocyte DNA is another mechanism that can increase telomerase expression and occurs in around 26%

of HBV related HCCs (Nault et al., 2013; Paterlini-Bréchot et al., 2003). In Asian HCC studies, Telomerase is upregulated by one of two mechanisms: $pTERT^{Mut}$ can occur, giving an extra binding site for the ETS, or HBV is inserted in to the telomerase promoter region which accounts for the lower rates of $pTERT^{Muts}$ seen in Asian populations (Nault et al., 2019).

Telomeric DNA can fold back on itself and a single stranded can invade the telomeric DNA, creating a t-loop, that is further protected from degradation by the Shelterin complex (Cesare & Reddel, 2010). This Telomerase-independent telomere protective mechanism occurs in up to 10-15% of all cancers and is called an alternative lengthening of telomeres (ALT) pathway that is now detectable (Zhang et al., 2019).

1.6.3.3 Telomerase Enzyme Detection

The Telomerase Repeat Amplification Protocol (TRAP assay) was originally developed by Kim et al to assess Telomerase activity (Kim et al., 1994; Singh et al., 2015). Essentially the TRAP assay measures Telomerase function by amplifying the Telomerase extension products using polymerase chain reaction (PCR) which are then detected by gel electrophoresis (Mender & Shay, 2015). As the amplification step greatly increases sensitivity of detection, this accounts for the fact that ~85-89% of fresh human tumour biopsies, and 98% of malignant cell lines, as well as ovaries and testes have detectable telomerase activity (Kim et al., 1994; Singh et al., 2015). Correlating telomerase activity with IHC data has found that cells with detectable Telomerase activity have a positive nuclear signal for TERT protein whilst those with undetectable telomerase activity do not (Singh et al., 2015).

A limitation of the TRAP assays being that either fresh tissues or cell lines are required for the purposes of analysis. Hence telomerase activity from archived FFPE material is currently unavailable for assessment.

1.6.3.4 Protein Expression

To be able to explore the potential use of Telomerase as a biomarker of tumour development, various detection methods are possible across a range of sample types. In peripheral blood samples these include ELISA and analysis of small extracellular vesicles (exosomes) amongst others. Serum detection with ELISA has been undertaken in non-small cell lung cancer patients with a lower probability of survival observed in patients who expressed Telomerase compared to non-expressers (Targowski et al., 2010).

More recent work has been undertaken when exploring the role exosomes play in carcinogenesis. Exosomes are small vesicles secreted by every tissue and found in all bodily fluids that were initially thought to be a waste product of cellular regeneration (P. Li et al., 2017). Telomerase positive exosomes can alter hTERT negative fibroblasts in to hTERT positive fibroblasts, thus demonstrating how these curious, subcellular vesicles can seed malignant characteristics in to a host cell (Gutkin et al., 2016). Whilst analysis of the exosomal expression of hTERT mRNA from serum has been described across a wide range of malignancies little is known of any role exosomal Telomerase plays in PLC, (Goldvaser et al., 2017).

Immunohistochemical and immunofluorescence detection of TERT has been undertaken in a wide range of tumour types, using a very diverse array of antibodies and detection methods. There is a lack of consensus in the published literature as to exactly what constitutes a positive control for TERT expression. Online repositories such as the Human Protein Atlas, and UniProt, state the RNA levels are detectable in lung, GI tract, Testis and lymphoid tissues (thymus, appendix, spleen, lymph node, tonsil and bone marrow) but protein levels are undetectable. Manufacturers of various monoclonal and polyclonal antibodies have not reached consensus the cellular and subcellular location of TERT expression for the purposes of a positive control. Some have stated that Tonsil

is a positive control, others have created genetically altered cell lines to overexpress TERT and use these as a positive control. There has been extensive evidence of the presence of TERT (by detection of the enzymatic activity, using the TRAP assay) that has been corroborated with Western blot data reporting similar detection in a variety of tumours, and in some adult tissues (Kim et al., 1994). Human testes appear to be one of the few normal human tissue types that have detectable TERT activity as well as detectable protein levels, that also stain positively in the nuclei of spermatocytes and maturing spermatids (Hiyama et al., 2001).

Owing to an incompletely understood physiological role of TERT, there is some evidence from the literature that other pools of Telomerase may well be present, outside the confines of the nucleus. Up to 20% of TERT is found in mitochondria and may migrate from the nucleus in states of oxidative stress to perform TERC-independent functions that are protective to the host mitochondria and increase tumour-cell survival (Chiodi & Mondello, 2012). This non-nuclear pool of TERT also complicates the staining of tumour tissues as they are otherwise indistinct from other extra-nuclear components, and can appear in the cytosol as well as the nucleus (Fujimoto et al., 2001; Y. Yang et al., 2002).

However, a small number of authors feel the detection of Telomerase using IHC may well be intrinsically flawed, owing to technical issues with the primary antibody (Y. L. Wu et al., 2006). Meaning that Telomerase IHC data should be *interpreted with caution* (Kim et al., 2013).

1.7 Exosomes

Exosomes were discovered as small vesicles jettisoned from maturing reticulocytes (Harding et al., 1983; Pan & Johnstone, 1983). The size of these vesicles being between 30-100nm and are part of the continuum of small particles of various sizes,

compositions, densities and bud from every cell and detected in every bodily fluid ever tested (Raposo & Stoorvogel, 2013). The importance of defining the origin of exosomes and differentiating them from their mimickers, microvesicles (MVs), is because the MVs can bud directly from the cell-membrane whilst exosomes are small packages of genetic material that come from the multi-vesicular endosome (MVE) that are endosomal in origin and are normally destined for lysosomal degradation (Raposo & Stoorvogel, 2013).

Hence there are some unique features of exosomes such as the presence of the trans-membrane *tetraspanins* (CD9, CD63, CD81, CD82) that have been used as a confirmatory biomarker as well as the lack of internal degradation machinery that allows for the presence of RNAs such as mRNA, microRNA and non-coding RNAs (Cheng et al., 2019). Other behavioural properties of exosomes include the ability to influence transcriptional activities of cells by the transfer of mRNA, e.g., resulting in detectable Telomerase proteins being found in non-malignant cells (Gutkin et al., 2016). Vesicles either fuse to the recipient cell's plasma membrane, or are endocytosed, thus allowing the transfer of the genetic material from the original cell to any recipient cell capable of uptake (Harding et al., 2013; Raposo & Stoorvogel, 2013). This ability to transfer genetic material from one cell type to another has been proposed as a mechanism of tumourigenesis. These unique characteristics allow exosomes to be exploited as potential biomarker source for a variety of cancers.

An increasing body of evidence is accumulating to implicate exosomes with epithelial to mesenchymal transition (EMT) as well as in cancer associated fibroblasts (CAFs) both of which are present in the tumour microenvironment (TME) (Chen et al., 2019).

1.7.1 Exosomal Survivin

Exosomal Survivin in detectable from cell culture and has even been implicated in exosomal internalisation, as blocking exosomal Survivin results in a reduction of internalised vesicles (Gonda et al., 2018). In cell culture models there is differential detection of Survivin in exosomes, depending on the cell line of origin. Survivin was the most commonly isolated IAP (by western blot analysis) when compared to XIAP, cIAP1, cIAP2 and Survivin as it was present in all six cell lines analysed (Valenzuela et al., 2015).

Tantalisingly, the potential locations Survivin can now be found in has expanded because of the exosomal proportion. This extracellular pool of Survivin confirms its status as a tumour associated antigen as it can modulate the TME and permit tumour growth (S. Khan et al., 2015). However there remains a considerable amount of work to record and reporting the role exosomal surviving may, or may not, play in human cancer growth.

1.7.2 Exosomal Telomerase

Given the lack of degradation machinery within exosomes, it is unsurprising that telomerase has also been reported in these small vesicular bodies. Cancer cell lines that express hTERT (and have detectable Telomerase activity) can also secrete exosomes have been transfected into hTERT-negative fibroblasts cells (Gutkin et al., 2016). The previously hTERT-negative somatic cells start expressing detectable telomerase activity within 24 hours (Gutkin et al., 2016). This is a truly remarkable finding and neatly demonstrates the ability of exosomes to seed immortality traits to other cells. The authors of the paper remarked on the increased replicative ability of these transfected somatic cells, and note the reversal of senescence in a small proportion of the cell populations.

This same group has expanded their work into a clinical cohort of 133 patients with various solid and haematological tumour types, not including liver cancers, and found varying levels of exosomal mRNA accordingly (Goldvaser et al., 2017). The serum-derived exosomes make for an ideal study material as serum samples are routinely taken for clinical assessment and do not require special processing or storage methods. Intriguingly the serum exosomal levels correlated well with the patients' disease progress, with a corresponding drop in detectable mRNA following curative surgical resection. Obviously, there is considerable work yet to be done in this field, as well as some novel research analysing the role exosomes play in PLC.

1.8 Project Aim

The aim of this project is to elucidate the role universal tumour antigens play in primary liver tumours. Assessing known genetic alterations in promoter sequences, and levels of transcribed UTAA RNA, and proteomic expression will all be explored and correlated with clinical data. Comparing UTAAs in tumour and background liver will allow me to ascertain whether genetic alterations are germline or somatic in nature. Whilst using paired samples for protein quantification will allow individual protein levels to be compared for each patient, as well as exploring correlations with clinical parameters.

Ultimately this project will confirm that UTA are indeed tumour-specific and find that promoter mutants, or protein levels can be used as a potential biomarker in future clinical practice.

2 Material & Methods

There is an inherent bias in studying tissues from patients that have been selected to undergo surgical treatment. This selection bias is unfortunately unavoidable, but every effort has been made to include samples from every patient that has been cared for at University Hospitals Plymouth since the hepatobiliary service commenced in 2005. As no previous research assessing hepatic neoplasms has been undertaken, at the basic science level, in the region this also has implications for the potential impact of the work. This exploratory descriptive work aims to initiate liver cancer research in the South West that will hopefully have local, regional, national and international implications for the greater understanding of hepatic carcinogenesis.

2.1 Archived Clinical Material

2.1.1 Ethical Stipulations

The Research Ethics Committee (REC) approval for use of the archived tissues for my research was given with support of the confidentiality advisory group (CAG) allowing Health Research Authority (HRA) approval for the project. To satisfy the CAG that this research would not inappropriately use data from patients, the Caldicott Guardian (Consultant Cardiothoracic Surgeon, Mr Adrian Marchbank) stipulated that every consent form signed by the patient prior to their operation must be visually inspected to ensure that each patient did not actively opt out of participating in research. There is a small box on the consent form the patient can mark should this be their wish. This was achieved with the help of the Research, Development and Innovation (RD&I) department at University Hospitals Plymouth as the overwhelming majority of medical records were still in the paper-based format. Notes were requisitioned reviewed in the RD&I offices. On the rare occasion that the consent form for the liver resection operation was missing from the archives this patient was excluded from further

participation. The opportunity to view the medical notes to review the consent form for each surgical procedure has facilitated a review of the past medical history of all of the patients who underwent surgery.

A requirement for ethical approval as stated by the REC is that I, the researcher, must be blinded to the origin of the donor tissues. Fortunately, helpful colleagues in the Department of Cellular and Anatomical Pathology at University Hospitals Plymouth were able to randomise the tissue samples, thus fulfilling this criterion. Different sample numbers were given for each research activity (i.e., DNA extraction and IHC) and in the order in which tissue blocks were processed. As such, there was no correlation between samples DNA 001 and IHC 001. If a repeat analysis was required (due to insufficient DNA extraction) then a new study number was issued to the repeat sample to minimise any other source of bias.

Finally, the use of tumour samples for research must not deplete the archive available for the potential use in future patient care. Meaning that larger specimens (resection tissues rather than biopsy samples) are more appropriate as the volume of tissue available for use is considerably larger than biopsy samples.

2.1.2 Auditing Potential Recruits

Once ethical approval for research was granted, it was possible to undertake an audit of all liver specimens held on the UHP databases. As the Hepatobiliary surgical service was established in 2005 this was the start point for recruitment purposes. As only larger surgical specimens will yield a sufficient volume of poor-quality liver tissues, these were the initial targets for use. However as there is no code recorded for ‘liver resection’ or ‘wedge resection of liver’ all liver tissues screened, hence manual checking of the database of samples was required.

All histopathological specimens receive a primary and secondary diagnostic code. In this case, the primary diagnostic code (T-62000) denotes that the tissues originate from the liver, the accompanying secondary diagnostic code is given for the tissue diagnosis (such as hepatocellular carcinoma) as outlined in table 2.

Number	Diagnosis	Secondary Code
445	Adenocarcinoma, metastatic, NOS	M-81406
369	Adenocarcinoma, NOS	M-81403
114	Hepatocellular carcinoma, NOS	M-81703
4	Hepatocellular carcinoma, fibrolamellar	M-81713
43	Cholangiocarcinoma	M-81603
23	Carcinoma, metastatic, NOS	M-80106
11	Carcinoma, NOS	M-80103
11	Adenoma, NOS	M-81400
11	Bile duct adenoma	M-81600
6	Liver cell adenoma	M-81700
6	Transitional cell carcinoma, NOS	M-81203
4	Small cell carcinoma, NOS	M-80413
3	Malignant melanoma, NOS	M-87203
2	Neuroendocrine carcinoma	M-82463
1	Clear cell adenocarcinoma, NOS	M-83103
1	Carcinosarcoma, NOS	M-89803
1	Squamous cell carcinoma, NOS	M-80703
1055	**Total**	

Table 2. The diagnostic codes for all liver specimens (biopsy and surgical resection) at Derriford Hospital from 2005 until September 2017.

Table 2 includes all biopsy and surgical resection specimens from 2005 until August 2017. Each individual sample also receives a unique sample number that can be searched on the hospital laboratory information management system (LIMS), which at UHP is iLab. The details held of iLab include the sample type (biopsy, surgical resection specimen) as well as descriptive terms for the tissue analysis, the size of the tumours and whether there is vascular or perineural invasion. An example to illustrate the importance of cross referencing the iLab reports is demonstrated in one case: a fibrolamellar HCC being coded as 'Carcinoma, NOS.' Whilst being labelled as a carcinoma is technically correct a more accurate method of recording the tumour types

is required for research purposes. The abbreviation ‘NOS’ refers to samples being ‘not otherwise specified.’

This method of screening has relied on correct coding by the reporting pathologist. However, this is a potential cause of bias at this screening stage as highlighted by one case being added to the database after the patient was met in follow up clinic, having had their tumour erroneously coded.

Further analysis of the archive search results separated out the surgical resection specimens from the biopsy samples. Surgical resection specimens FFPE tissues are typically 15x10mm in size, which is a significantly larger amount of tissue than can be provided from a single needle biopsy. As mentioned above: biopsy samples were felt to be inappropriate for use in research as their inclusion and processing may deplete the tissue archive completely.

Searching through the euphemistically coded ‘Adenocarcinoma, NOS’ and ‘Carcinoma, NOS’ yielded further cases of primary liver neoplasms. The decision tree can be seen in figure 4 and outlines how the 1055 samples were reduced to samples that could be used in research – the colour red denotes excluded samples.

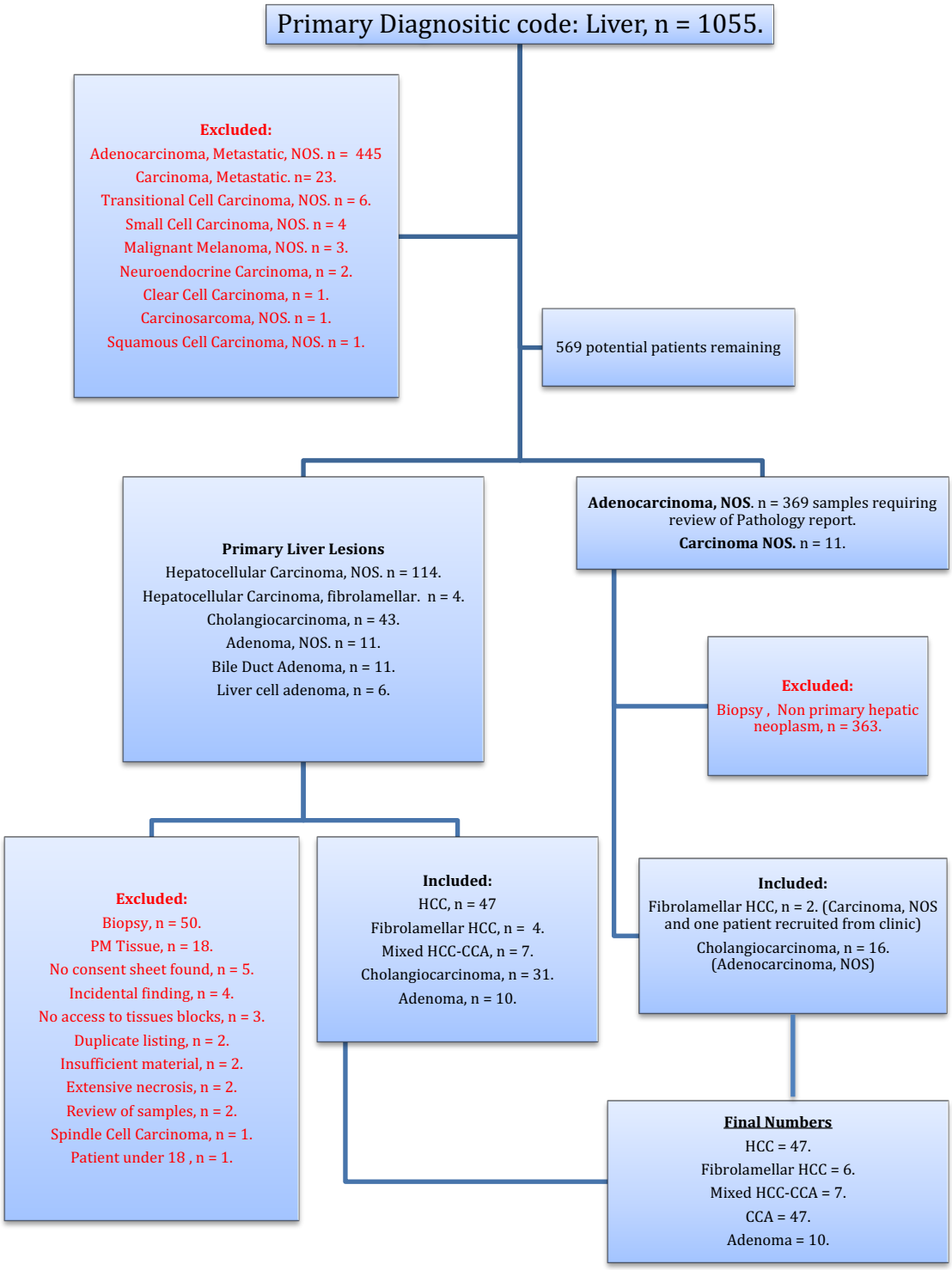


Figure 4 All 1055 samples from the tissue archive at UHP between 2005 & 2017.

Upon closer review of the 117 resection specimens in figure 4, 11 of the CCA were distal extrahepatic tumours, and therefore excluded. Five further HCC cases were

recruited prospectively from clinic, resulting in 52 HCCs as well as 6 FL-HCC. All of the potential primary liver neoplastic surgical resection specimens have been recorded with the culmination of this advanced screening being shown in table 3.

Neoplasm	Number
Hepatocellular carcinoma	52
Fibrolamellar Hepatocellular carcinoma	6
Mixed Hepatocellular-Cholangiocellular carcinoma	7
Cholangiocellular carcinoma	36
Adenoma	10
Total	**111**

Table 3 Surgical resection specimens of primary liver neoplasms by tumour sub-category.

2.2 Prospectively Recruited Patients

Seventy-four patients have been recruited into the Inflammatory Liver Biobank (REC reference: 15/LO/0948), including two cases of follow up bloods when a second set was taken prior to a second intervention. Five of these prospectively recruited patients underwent surgical resection for HCC and have been included in the final study figures in table 3. Of the 74 patients, 49 samples were taken from patients undergoing treatment for a primary hepatic pathology with the remaining 26 samples from patients who had surgical excision of a colorectal metastasis. Control groups include both healthy volunteers (n=10) and stable cirrhotic patients under six monthly HCC-surveillance (n=10).

2.3 Research Cohort

The research cohort for tumour resections includes 111 patients is shown in table 4. It is worthwhile to remember that there are selection biases due to these patients being offered an operation and that were deemed medically fit enough to undergo this treatment. Clinical follow up includes patients who died within one month of their operation. Viral hepatitis per tumour category: HCC (one dual Hepatitis B&C, 7

Hepatitis C, one Hepatitis B), CCA (one each Hep B and C), Mixed tumour (one dual Hep B&C, 3 Hep C). Metabolic risk factors include the clinical characteristics diabetes mellitus, and obesity in addition to histological features: NAFLD & steatosis.

Neoplasm	HCC	FL-HCC	CCA	Mixed	Adenoma
Total	52	6	36	7	10
Male	40 (76.9%)	4 (66.7%)	16 (44.4%)	5 (71.4%)	1 (10.0%)
Female	12 (23.1%)	2 (33.3%)	20 (55.6%)	2 (28.6%)	9 (90.0%)
Age (range)	67 (41 - 84)	33 (22 - 42)	64 (44 - 83)	60 (47 - 72)	37 (27 - 51)
Months follow up (range)	47 (0 - 130)	94 (21 - 159)	35 (0 - 129)	31 (3 - 76)	88 (30 - 138)
Fibrotic	26 (50.0%)	0 (0.0%)	9 (25.0%)	6 (85.7%)	3 (30.0%)
Cirrhotic	16 (30.7%)	0 (0.0%)	1 (2.8%)	6 (85.7%)	0 (0.0%)
Viral Hepatitis	9 (17.3%)	0 (0.0%)	2 (5.6%)	4 (57.1%)	0 (0.0%)
Metabolic Risk Factors	28 (53.8%)	0 (0.0%)	15 (41.7%)	3 (42.9%)	8 (80.0%)

Table 4. Clinical characteristics of the study cohort.

Regrettably the medical records for biomarkers (such as AFP, CA19-9 and CEA) were incomplete so this data has not been included. Of the 58 HCCs, at least 19 were missing an AFP, and of the 36 CCAs there was no record of an AFP value in at least 14 cases and no recorded CA19-9 from a minimum of 23 patients.

2.3.1 Haematological Samples

Four vacutainers of serum (BD Vacutainer® SST™ II Advance, Thermo Scientific™) and two EDTA (BD Vacutainer® K2E 7.2mg, Thermo Scientific™) blood samples were taken from each patient. The samples were gently inverted 10 times after venesection with the serum samples requiring 30 minutes at room temperature. After inversion, EDTA samples were immediately stored at 4 degrees Celsius until further processing. Both serum and plasma samples were then centrifuged at 2500g for 10 minutes at 4 degrees Celsius. Plasma and Serum samples are stored in 1ml aliquots; buffy coat and red blood cells are stored separately in various volumes, determined by the amount available in each individual sample. All blood samples are stored in 2-dimensional barcoded blood tubes (1.0ml External Thread Next-Gen Jacket Tube with 2D code on

Tube Base & Linear Barcode, HRN on side, with Screw Cap, Brooks Automation Ltd) all of which are kept at minus 80 Celsius.

2.3.2 Tissue Samples

Regrettably, there was no provision in place at UHP for sampling fresh tumour for research purposes. Space in the Histopathology department was deemed insufficient to allow for a 'hot desk' to be used for sample cut up on an *ad hoc* basis as, and when, research samples came from theatres. FFPE tissue samples from patients who were prospectively recruited were processed in the same manner as the archived tissue samples.

2.4 DNA from FFPE Tissues

In the National Health Service formalin fixed paraffin embedded (FFPE) tissues are kept for 30 years after removal from a patient. Given the huge archive of tissues a single hospital would create, and the associated inventory costs, it has become increasingly popular to store samples off site, more recently for UHP samples, in a climate-controlled site in mid-Wales. Tissue blocks that could potentially be used for research have clinical details such as tumour size, tissue morphology and surgical resection margin stored indefinitely on the digital databases. These archives can be searched for by their individual case number on the iLab system to assess their suitability for use in research, as well as recording clinical details from the formal histopathological reports. Characteristics such as size of resection specimen can be cross-referenced to ensure only samples of adequate size are included for research. The tissue blocks are shipped from the storage unit to UHP by a hospital-approved courier.

To avoid any potential breach of confidentiality the tissue blocks are stored and processed within the Department of Cellular and Anatomical Pathology at UHP. Blocks from various parts of the tumour or background liver were each given a unique

designation when being prepared with the key to this is readily available on the database.

Using a microtome (Leica RM 2135) in the Pathology Department at University Hospitals Plymouth, 5µm slices of tissue were cut from the tissue blocks. The first few slices were discarded to avoid oxidative damage affecting the tissues under analysis. Three 10µm curls of tissue are taken from the FFPE tissue block and were sealed in sterilised 1.5ml Eppendorf tube immediately. Tissue from the tumour, as well as background tissue (where possible) from the same patient was taken to explore any differential expression between the background liver tissue and the any neoplastic growth. To reduce the chance of contamination between samples; gloves were worn throughout the handling process, the cutting blade (Thermofisher Scientific MX35 ultra) changed for every tissue block and any surface that has come into contact with the cut tissue was cleaned with 90% alcohol between tissue blocks. Tissue curls were sealed in sterile Eppendorf tubes, figure 5. The liver tissue is generally brown in colour with the surrounding paraffin being white.

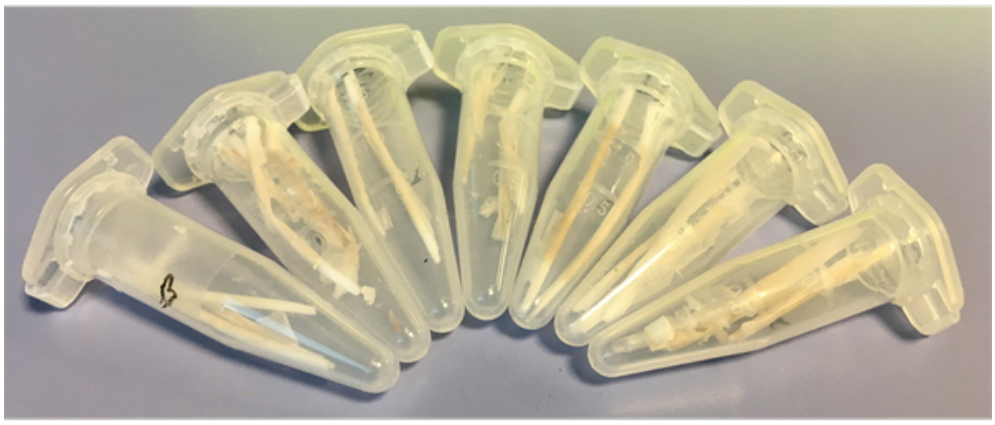


Figure 5. Eppendorf tubes containing tissue curls extracted from FFPE archived materials.

FFPE tissue curls were transported to the university research labs and the commercially available kit (QIAamp DNA FFPE Tissue Kit, Qiagen) was used to extract DNA from these samples following the protocol from QIAamp DNA FFPE Tissue Handbook, as

outlined briefly below. Buffers AL and ATL were heated to 70 degrees Celsius in a water bath to dissolve precipitates.

Deparaffinisation was undertaken with 1ml of xylene and vigorous vortexing (ZX3 Vortex Mixer, Fisherbrand) for 10s, following by centrifugation at full speed (Centrifuge 5418, Eppendorf) for two minutes at RT. Occasionally a small amount of tissue remained unprocessed and was filtered out during centrifugation steps, as outlined in fig 6. The presence, or otherwise, of this material did not impact on subsequent results obtained.

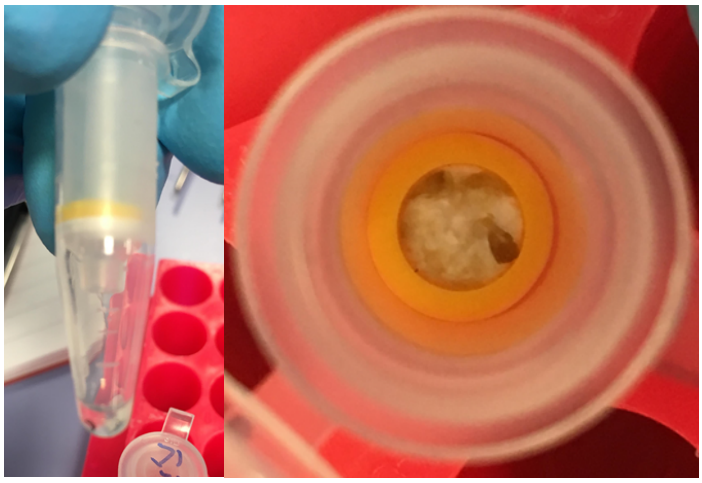


Figure 6 Spin columns used for DNA extraction from FFPE tissues. The clear eluate in left image. Some tissue remains in the filter after processing in the right image.

The supernatant was removed by pipetting with care taken to ensure the pellet remained undisturbed and 1mL of ethanol (purity > 99.8%, Honeywell) was added to the tissue pellet. The vortexing, centrifuging steps and supernatant removal steps were repeated to remove the ethanol. Residual ethanol was encouraged to evaporate as the Eppendorf lid was left open and the samples were heated to 37 Celsius in a heat block for ten minutes. The tissue pellet was then re-suspended in 180µL of Buffer ATL, with the addition of 20µL of proteinase K and briefly mixed by vortexing. This mixture was then incubated at 56 Celsius in a heat block for one hour, followed by a further hour incubating at 90 Celsius for another hour to partially reverse formaldehyde modifications.

The samples were cooled to RT and briefly centrifuged to remove drops from inside the lid. Buffer AL (200µL) was added, mixed by vortexing, and followed by the addition of 200µL of ethanol and further, brief, vortexing. The lysate was again briefly centrifuged to remove droplets from inside the lid and was then transferred into a spin column within a 2mL collection tube. This spin column was then centrifuged at 6000g (8000rpm) for one minute, with the flow through/eluate discarded. Wash steps (Buffer AW1 then AW2) were undertaken with the addition of 500µL of wash buffer AW1, followed by centrifugation at 6000g and discarding the flow through. The process was repeated with the addition of 500µL of wash buffer AW2. The spin column membrane was then dried by centrifuging at 20,000g/14,000rpm for three minutes in a new, sterile, 2mL collection tube. The column was then placed in a sterile 1.5mL Eppendorf with 80µL of Buffer ATE (elution buffer) used in the elution step and allowed to equilibrate for five minutes at RT. Finally, the sample was spun at full speed (20,000g/14,000rpm) for one minute with the eluate being kept. Two microlitres of eluted DNA was placed on the NanoDrop™ 2000 spectrophotometer (Thermo Scientific™) to assess both the quality (260/280 ratio) and quantity (concentration, in ng/µL) of extracted DNA. Samples were labelled and stored at -20 Celsius until required.

2.5 Sequence Amplification

The published literature was searched for primer pairs that had been successfully used for the polymerase chain reaction (PCR) targeting short DNA sequences of the promoter regions. The information is outlined below in tables 5 & 6. Which can be compared to a recent meta-analysis that reports the use of PCR-RFLP or TaqMan assays, rather than Sanger sequencing, as the preferred method of detection for Survivin SNPs (Moazeni-Roodi, Ghavami and Hashemi, 2019). As can be demonstrated from table 5, the preferred source of DNA for assessing the Survivin promoter is whole blood, rather than FFPE tissues.

Tissue of Origin (reference)	DNA Source	PCR Product (base pairs)	Analysis Method	Primer Name
Wilms Tumour (Radojevic-Skodric et al., 2012)	FFPE tissue	151	RFLP	Surv F1 R1
Gastric Cancer (Yang *et al.*, 2009)	Whole blood	151	RFLP	Surv F1 R1
Urothelial Cancer (Wang *et al.*, 2009)	Whole blood	341	RFLP	Surv F2 R2
Breast Cancer (Rasool *et al.*, 2017)	Whole blood	341	RFLP	Surv F2 R2
HCC (Bayram *et al.*, 2011)	Whole blood	341	RFLP	Surv F2 R2
Ovarian Cancer (Han *et al.*, 2009)	Whole blood	329	RFLP	Surv -31 F3 R3 Surv -625F + R
Colorectal Cancer (Yamak *et al.*, 2014)	FFPE tissue	329	RFLP	Surv -31 F3 R3 Surv - 241 F + R Surv -625

Table 5 Research resources for Survivin -31G/C SNP detection.

Sanger sequencing the PCR product of the pTERT region was more commonly undertaken, as well as using a variety of sources of sample DNA, as shown in table 6.

Tissue of Origin (reference)	DNA Source	PCR Product (base pairs)	Analysis Method	Primer Name
Glioblastoma Multiforme (GBM) (Purkait *et al.*, 2016)	FFPE & Frozen	163	Sanger	Short TERT
Malignant Melanoma (Horn *et al.*, 2013)	Whole blood	163	Sanger	Short TERT
Low Grade Glioma (Chan *et al.*, 2015)	FFPE	163	Sanger	Short TERT
13 Tumour types (Huang *et al.*, 2015)	FFPE	190	Sanger	TERT F3 R3
Follicular Thyroid Adenoma (Wang *et al.*, 2014)	Frozen	NS	Sanger	hTERT Promo F + R
Bladder Cancer, Glioblastoma. (Liu *et al.*, 2013)	NS	235	Sanger	hTERT Promo F + R
HCC & HCAs (Nault *et al.*, 2013)	Frozen	163	Sanger	Short TERT
Bladder Cancer (Pivovarcikova *et al.*, 2016)	Voided Urine and FFPE	NS	NGS and Sanger	hTERT Promo F + R

Table 6 Research resources for determining Telomerase promoter region SNP detection.

2.5.1.1 Initial Work

Oligonucleotides were ordered in and trialled using CRC FFPE tissues as the template DNA, with the polymerase (Dreamtaq) that had been used by other members of the lab undertaking PCR with cell culture samples. When creating a mastermix for n samples, enough reaction volume was made for n+2 reactions, to ensure enough reaction mixture for a no-template negative control and to allow for pipetting error. Initial PCR preparation was undertaken on the bench top, but this was quickly moved to a laminar-flow hood to avoid contamination of constituent components and thus erroneous results.

The initial reaction mixture, as shown in table 7.

Component	**Volume for one 50µL reaction**	**Final concentration**
DreamTaq	5 µL	1X
dNTPs (2µM)	5 µL	0.2µM
Forward Primer (10µM)	0.5 µL	0.1µM
Reverse Primer (10µM)	0.5µL	0.1µM
Template (max 200 ng)	1µL	As required
Polymerase	0.25µL	1 U
Ultrapure, distilled water.	37.75µL	-

Table 7. Reagents for PCR using DreamTaq polymerase.

With reaction conditions as outlined in Table 8 using the Labtech GS4 G-Storm, Gene Technologies Thermal Cycler:

Step	**Temperature (Celsius)**	**Time**
Initial denaturation	95	3 minutes
40 cycles of steps 1-3:		
1. Denature	95	30 seconds
2. Anneal	52	30 seconds
3. Extend	72	1 minute
Final extension	72	5 minutes
Hold	4	Indefinite

Table 8. Reaction conditions for PCR using DreamTaq polymerase.

PCR products were run on a 2% Agarose gel with a 100base pair ladder (Gene Ruler, ThermoFisher) and loading dye (Loading Dye 6X Orange DNA, ThermoFisher) and distilled water. Small volumes of PCR product (4 µL) were mixed with loading dye (Loading Dye 6X Orange DNA, ThermoFisher) and run alongside to assess the size of PCR product. Optimisation for Dreamtaq included altering magnesium concentrations as well as the quantity of template DNA added and gradient PCR.

2.5.2 Optimisation

2.5.2.1 Primer Selection

There was poor reproducibility and results from extensive work with longer PCR products (>200 base pairs) so efforts were concentrated on targeting shorter sequences in the promoter regions of both Survivin and Telomerase. There were also a number of potential target sequences but regions around Survivin -31 and Telomerase -124 (C228T) & -146 (C250T) were focussed on. Given that most DNA taken from FFPE tissues is of a poor quality and fragmented at around 250 base pairs, there were limitations on the selection of potential primer pairs (Dedhia *et al.*, 2007).

2.5.2.2 Gradient PCR

The primer sequences (composed of nucleotides G, C, T and A) were known, but early work found that some of reactions needed further optimisation. Approximate annealing temperature (T_a) for the primers selected for PCR was estimated using the calculated melting temperature (T_m) in degrees Celsius as demonstrated by the equations below:

$T_m = 4(G+C) + 2(A+T)$.

Also;

$T_a = T_m - 5$

As previously used (Roux, 2009).

The T_a was used as the mid-point for the gradient PCR to assess various annealing temperatures simultaneously. There was also the opportunity to focus on smaller temperature steps (less than one degree Celsius) or create a grid of temperatures and alter a secondary component of the reaction, like magnesium concentration, as shown in figure 7, (Najafov *et al.*, 2017).

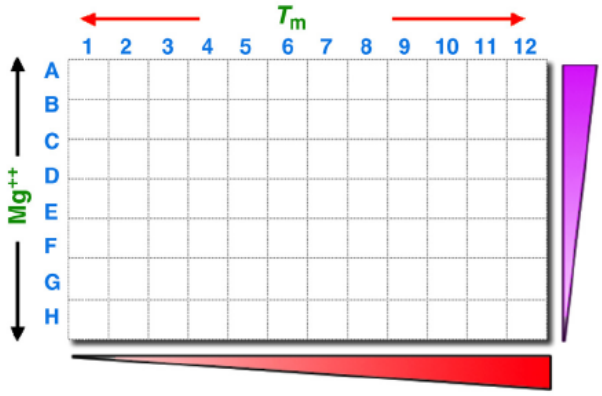


Figure 7. A grid pattern for altering two components of PCR simultaneously.

2.5.2.3 Template DNA

The quantity of template DNA used in the PCR reaction was polymerase dependent, as advised by the manufacturers. Generally, between 100 – 200ng of DNA was added to the PCR reaction, and only in exceptional circumstances, such as repeated failures of results, was this amount of template DNA increased.

2.5.2.4 Enzyme Selection

Owing to the relatively short and frail nature of the FFPE DNA, not all of the commercially available polymerase enzymes were able to amplify certain primer pairs. For Survivin the Accuprime™ Pfx polymerase (Thermo Scientific™) yielded the highest success whilst for the Telomerase promoter region the best candidate was the KAPA2G Fast Hotstart ReadyMix polymerase. Other enzymes that were trialled included: Q5 High Fidelity Polymerase (New England Biolabs), Accuprime™ Taq DNA Polymerase, High Fidelity (Thermo Scientific™).

2.5.2.5 Universal Primers

In an attempt to increase the read length of the PCR product for Survivin (151 base pair sequence) a universal primer was added on to the 5' end of the primers (Kelley and Quackenbush, 1999). The increase in size of the universal primer would, theoretically, have resulted in a PCR product that was longer than the original sequence by the same number of base pairs. Sadly, the quantities of PCR product that resulted were too low (i.e., significantly below the 100ng minimum required) to allow for Sanger sequencing.

Initial results from sequences were delivered using primers for the 151 base pair sequence the chromatograms demonstrated the region of interest being as little as 20 bases from the start of the sequence. In an attempt to increase the read length, universal primers were added on to the 5' end of the original oligonucleotides to lengthen the PCR product.

The two universal primers chosen (M13-21 and M13-29) did not cause any primer-dimer or form hairpin loops (due to inverted repeats) when run on online software platforms such as OligoEvaluator (Sigma-Aldrich) and Multiple Primer Analyzer (Thermo Scientific). The oligonucleotides that were used are outlined below in table 9.

Primer Name	**Oligonucleotide Sequence**
Survivin 31 F1	AAGAGGGCGTGCGCTCCCGACA
Extended F1 M13-21F (Ext. F1)	TGTAAAACGACGGCCAGTAAGAGGGCGTGCGCTCCCGACA
Survivin 31 R1	GAGATGCGGTGGTCCTTGAGAAA
Extended R1 M13-29R (Ext. R1)	CAGGAAACAGCTATGACCGAGATGCGGTGGTCCTTGAGAAA

Table 9. The addition of universal primers (Extended F1/R1) to the underlined oligonucleotide sequence for a primer pair, Survivin F1 R1, that have been previously used in assessing the Survivin promoter region.

Underlined regions in the above table highlights the same oligonucleotide sequence with the addition of the universal primer at the 5' end of the sequences, which are not

underlined. Gradient PCRs were undertaken with the added universal primers using the annealing temperatures of between 60 and 72 Celsius.

2.5.3 Survivin Promoter

The reaction volume was dictated by the polymerase used for PCR, as such for Accuprime a reaction volume of 50uL was required. Master mixes were created on ice and included an additional volume to allow for pipetting error. Each mastermix was created for 12 reactions, which included a no template negative control for quality assurance. For each reaction the following quantities were used, as advised by the manufacturer and is shown in table 10.

Component	Volume for one 50µL reaction	Final concentration
10X AccuPrime Pfx Reaction Mix	5 µL	1X
Forward Primer (10µM)	0.75 µL	0.15µM
Reverse Primer (10µM)	0.75µL	0.15µM
Template DNA (max 200 ng)	2µL	As required
AccuPrime Pfx DNA Polymerase	0.4µL	1 U
Ultrapure, distilled water.	41.1µL	-

Table 10. Reagent for the Accuprime Pfx polymerase for PCR.

For optimisation of the reaction for maximal PCR product, a gradient PCR was undertaken using the same template DNA and reaction mixture in one thermal block. There was a heated lid, maintained at 112 Celsius. Once the thermocycler had completed the thermal block was held at 10 Celsius until the PCR products were placed on ice or at 4 Celsius. Cycling conditions can be found in table 11.

Step	Temperature (Celsius)	Time
Initial denaturation	95	2 minutes
35 cycles of steps 1-3:		
1. Denature	95	15 seconds
2. Anneal	52 - 64	30 seconds
3. Extend	68	1 minute
Final extension	68	5 minutes

Table 11. Reaction conditions for gradient PCR targeting the Survivin promoter region, using the Accurprime Pfx polymerase.

Following the reaction conditions outlined in table 11, the optimal annealing temperature for the Survivin promoter was found to be 62 Celsius.

2.5.4 Telomerase Promoter

As the Accuprime Pfx polymerase enzyme was successful in sequencing FFPE DNA from the Survivin promoter region, it was trialled with primers targeted to the Telomerase promoter region with disappointing results. Despite considerable attempts at optimisation there was a distinct lack of single clear bands when viewed after gel electrophoresis. Gradient PCR, Touchdown PCR and variable concentrations of Magnesium yielded poor results.

When trying to recapitulate the experiment to sequence the promoter region of Telomerase, having had difficulties with alternative polymerase enzymes, the KAPA2G enzyme was purchased. Given that it was used in two separate publications using two different primer combinations it was felt that exploring this as a potential polymerase was essential (Chan *et al.*, 2015; Huang *et al.*, 2015).

The reaction volume required for the KAPA2G polymerase was 25µL. Mastermixes were also made for a total of 12 reactions with one volume for the no template negative control and one volume spare. The reaction volumes are outlined below, as advised by the manufacturer, in table 12.

Component	Volume for one 25µL reaction	Final Concentration
2X KAPA2G Fast Hotstart ReadyMix	12.5µL	1X
Forward Primer (10µM)	1.25µL	0.5µM
Reverse Primer (10µM)	1.25µL	0.5µM
Template DNA	1µL	As required, <100ng total
Ultrapure, distilled water	9µL	-

Table 12. Reagents for the KAPA2G Fast Hotstart PCR.

Gradient PCR was undertaken with two sets of primers (yielding 163 and 190 base pair products) using the KAPA2G polymerase. Initial temperatures for the annealing steps were between 56.1 and 63.1 Celsius in ~1-degree increments. A more focussed gradient PCR analysing annealing temperature steps 60.1 - 63.9 Celsius was undertaken to clarify the best annealing temperature and can be seen in table 13.

Step	Temperature (Celsius)	Time
Initial denaturation	95	3 minutes
45 cycles of steps 1 - 3.		
1. Denaturation	95	15 seconds
2. Annealing	56.1 - 63.1	15 seconds
3. Extension	72	1 second
Final extension	72	10 minutes.

Table 13 Reaction conditions for a gradient PCR targeting the Telomerase promoter region.

Reaction mixtures for Telomerase promoter gradient PCRs. After successfully producing enough PCR product to send for sequencing, the following final reaction conditions were used for all following reactions.

Following optimisation, the best annealing temperature was found to be 60 Celsius. Once the Thermocycler had completed the program the thermal block was held at 10 Celsius until the PCR products were placed on ice or stored at 4 Celsius.

2.5.5 PCR Quality Assurance

A 2% Agarose gel (Topvision Agarose Thermo Scientific™) was used to confirm the presence of the correct size of amplification product using added SYBR™ Safe DNA Gel Stain (Thermo Scientific™) using a 100 base pair DNA ladder Generuler 100BP Plus (Thermo Scientific™) and analysing with the gel imager (Labtech GS4 G-Storm, Gene Technologies Thermal Cycler) (*Promega Guide to Agarose Gels.*, 2018). Gels were made with 1X TAE buffer and loaded with a small volume of PCR product: 6 µL for Survivin (with 1uL of added loading dye 6X Orange DNA loading dye, Thermo Scientific™), and 3 µL for Telomerase promoter PCR products. No additional loading dye was required for the KAPA2G reactions as it was already included in the reaction mixture.

A clean, single band on the gel was required for sequences to be processed further providing the no-template negative control remained did not demonstrate a band, thus implying there was no contaminants in the mastermix.

2.5.5.1 PCR Purification

Once the PCR product has demonstrated a single, clean, band on a 2% agarose gel, it was concentrated for ease of sequencing. The PCR products were purified using a DNA clean and concentration kit (DNA Clean & Concentrator-5 Zymo Research) as outlined briefly below.

In a 1.5mL Eppendorf, five volumes of DNA binding buffer was added to one volume of PCR product and briefly vortexed and centrifuged to remove contents from the inside of the lid. The mixture was then transferred to the spin column, which was placed inside a collection tube and centrifuged at full speed for 30s. 200µL of wash buffer was added to the column and centrifuged at full speed for 30s. The flow-through was discarded and the wash step was repeated again. Finally, 15µL of distilled water was added to the

column matrix and incubated at RT for one minute. The spin column was transferred to a sterile 1.5ml Eppendorf and centrifuged at full speed for 30s to elute the PCR product. The Quality and quantity of this purified PCR product was then assessed using a 1.5µL volume (NanoDrop™ 2000 spectrophotometer Thermo Scientific™). Only samples with 260/280 ratios greater than 1.8 were used for further analysis.

2.5.5.2 Sanger Sequencing

To enable bi-directional sequencing, 2µL of the forward primer (10nM) was added to 100ng of purified PCR product in a sterile 1.5mL Eppendorf tube. The addition of sterile nuclease/RNase free distilled water was added to give a final volume 14µL. This was process was repeated with the reverse primer to enable bi-directional sequencing from a single, purified, PCR product. A barcode label was placed on each sample, provided by LGC Genomics GmbH, and these were then posted for Sanger Sequencing.

2.5.5.3 Sequence Analysis

Once the sequenced files were made available by LGC, a genome database was searched for similar sequences and this demonstrated that the correct target had been amplified. The software package, *Macvector*, has been used to check the quality of the sequences and find the region of interest (ROI) with confirmation of sequence requiring bi-directional concordance. Visual inspection of the chromatograms was undertaken, with evidence of contaminated and uncontaminated samples shown in figure 8.

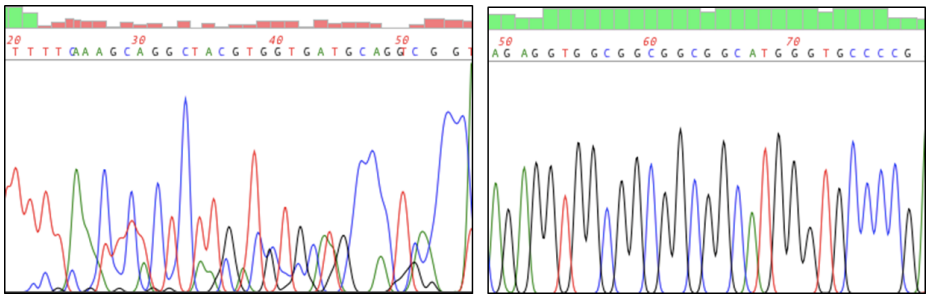


Figure 8. Chromatograms from MacVector with evidence of contamination (left) compared to an uncontaminated sequence. Note the low confidence (red bars, >1% error in the left image) compared to the high-confidence (green bars, <1% error in the right image) in base calls.

Chromatogram data was compared to reference database (Genome Reference Consortium Human genome build 37, GRC37) to assess for polymorphisms in the ROI. GRC37 was used in favour of GCR38 as the SNPs referenced throughout the literature in the Telomerase promoter region remained in their respective positions (-124 and -146 upstream of the transcription start site) instead of the right-shift that is understood to have taken place with later editions of GRC (National Centre for Biotechnology Information, 2017).

2.6 Immunohistochemistry

Formalin Fixed Paraffin Embedded (FFPE) tissue sections were cut at 4 μm by the Department of Cellular and Anatomical Pathology at University Hospitals Plymouth and mounted on positively charged glass slides. Optimisation of primary antibody was performed on various non-liver tissue sections. For Survivin, the positive control was human tonsil, for Telomerase normal testis was used. Negative controls were run in parallel with the positive control and normal liver by omission of primary antibody.

The indirect staining method with the avidin-biotin complex and 3,3′-Diaminobenzidine (DAB) tissue staining, with Mayer's haematoxylin as counterstain, for assessment of tissue expression of tumour antigens.

2.6.1 Antibody Selection

Online resources, such as Biocompare, as well as the published literature were searched for potential primary antibodies for IHC. Preference was given to antibodies that have previously been published using immunohistochemistry on paraffin (IHC-P) tissues. The results can be seen in table 14.

Antibody	Advised Dilutions	Isotype	Citations + Reference
Survivin (71G4B7), Cell Signalling Technologies (#2808s).	1:400	Rabbit Monoclonal IgG	219, (de Graaff *et al.*, 2017)
TERT Antibody 2C4, Novus Biologicals. (NB100-317).	1:50	Mouse Monoclonal IgM	18, (Lotfi *et al.*, 2014)
Anti-Telomerase reverse transcriptase antibody, C-terminal, Abcam (ab183105)	1:50 – 1:200	Rabbit Polyclonal IgG	8, (Yang *et al.*, 2017)
TERT Antibody (A-6), Santa Cruz Biotechnology (sc-393013).	1:50 – 1:500	Mouse Monoclonal IgG2b	4, (Zhou *et al.*, 2017)

Table 14. Published literature was consulted to screen potential IHC antibodies for detecting Telomerase and Survivin in human tissue samples.

2.6.2 Optimisation

2.6.2.1 Selection of Positive Controls

Using FFPE tissues allows for the assessment of cellular morphology as well as being able to stain for various proteins and assessing their expression in various subcellular localisations. To ensure appropriate positive and negative controls were used in staining a mixed approach was used in the assessment of potential positive controls.

Manufacturers tend to include a putative positive control in their literature, but Torlakovic and colleagues have stated that these can be "both imprecise and inadequate in providing the information needed" for reproducibility in the assessment of sensitivity and calibration of IHC staining (Torlakovic *et al.*, 2015). Thus, a combination of methods was used to ascertain a true positive control, given that intracellular location would, hopefully, vary across tumour grades and stages.

Online resources, such as Uniprot and The Human Protein Atlas (HPA) were used in the first instance. The vast quantities of data available on the HPA reference a wide range of common tumour and tissue subtypes, but this is not exhaustive, as many cellular and subcellular expression patterns remain undocumented (Cornish *et al.*, 2015). An idea of the expected cellular expression and subcellular location of staining in tissues can be

found by combining both online resources and the published literature as shown in table 15.

Target	Antibody	Expected Tissue Expression	Expected Intracellular location	Evidence Source
Survivin	Survivin (71G4B7) Rabbit Mab IgG	Tonsil, Male Tissues, GI Tract.	Nuclear in Germinal Centre Cells (GCC) >> Non GCCs.	The Human Protein Atlas
Survivin	Abcam, EP2880Y.	Tonsil, NOS.	Nuclear	Journal, (Varughese, Skjulsvik and Torp, 2017)
Telomerase	Abcam, ab183105.	Human sarcoma, glioma, tonsil, ovarian and thyroid cancer tissues.	Nuclear	Manufacturer's website & Journal (Zou *et al.*, 2016)
Telomerase	Thermo Fisher Scientific 2C4 clone, MA5-16034	Human Tonsil	Nuclear	Journal, (Carkic *et al.*, 2016)
Telomerase	Bio-Techne NB100-317	Not stated	Nuclear/Nucleolar	Manufacturers & publications (Lotfi *et al.*, 2014; Biron-Shental *et al.*, 2016)
Telomerase	Santa Cruz A6 Ab C12 Ab	Lymph node, Tonsil. Tonsil, Paediatric Thymus.	 Nuclear Nuclear	Manufacturers & publication (Zhou *et al.*, 2017)

Table 15. Published positive controls for IHC.

Confidence in the Survivin antibodies was boosted by their significant use in peer-reviewed publications. However, Telomerase antibodies have fewer references and there is less consensus on exactly what tissues should stain positive for this protein. Online resources state that there should be no expression of Telomerase in Tonsillar tissues, yet this is regularly cited as an appropriate positive control.

Combining online resources and the literature allows clarifies the detection of Telomerase in tissues (such as testis, ovary, placenta) with western blot and confirms the functionality of the telomerase enzyme using the telomerase repeat amplification assay (Kim *et al.*, 1994). Optimisation of potential antibodies was undertaken in these potential tissue types.

2.6.2.2 Retrieval Buffer

For the purposes of optimisation both Tris-EDTA and Citrate buffers were trialled to ascertain which gave the best results. Tris-EDTA 10X concentrated stock was made by adding 24g of Triz (Sigma-Aldrich®) and 2g of EDTA (Sigma-Aldrich®) to 1L of deionised water at pH 9, using 5M HCl (Sigma-Aldrich®). A 1:10 dilution was made with distilled water for use in the antigen retrieval stage of IHC.

Citrate buffer 1X working stock was made with the addition of 2.1g Citric acid (Sigma-Aldrich®) to 1L deionised water at pH 6 using 5M NaOH (Sigma-Aldrich®).

2.6.2.3 Primary Antibody Dilutions

The Survivin primary antibody was diluted to 1:400 in 1X TBS-T. The Telomerase antibody was also diluted to 1:400, but in a 2% Goat serum (v/v) TBS-T.

2.6.2.4 Blocking Steps

A number of blocking steps were used in the optimisation process to reduce background staining. Endogenous peroxidase was blocked with 3% Hydrogen Peroxide (Fisher Scientific) in Methanol (VWR®).

For routine staining, a second blocking step was used. Serum from the host species of the secondary antibody was used in a 1:100 dilution in TBS-T. One drop (50uL) of serum was added to 5mL of TBS-T and gently mixed by vortexing and 150uL was added to each slide mounted section of tissue. Using serum from the host secondary (Horse for Survivin, Goat for Telomerase) can help reduce background staining (Ramos-Vara, 2005).

To block nonspecific binding of endogenous Biotin, samples were incubated sequentially with Avidin D solution and then with a Biotin solution (both: Vector Laboratories) to give a near-irreversible binding in the Avidin-Biotin complex (Bratthauer, 2010).

In an attempt to reduce background-staining, serum from the host species for the biotinylated secondary was diluted to a concentration of 2% (v/v) when diluting the Telomerase primary antibody. Alternatively, the Survivin primary antibody was diluted in TBS-T.

2.6.2.5 Biotinylated Secondary & Detection

An IgG specific universal kit was purchased and used in the detection of the primary antibody. The Vectastain Elite ABC HRP Kit (Peroxidase, Universal. Vector Laboratories) was used in the detection of Mouse/Rabbit IgG with horse anti-rabbit /mouse secondaries and normal horse serum used for this isotype of antibody. For Telomerase a Mouse anti human IgM primary antibody, a separate biotinylated secondary (Goat anti mouse) and serum (goat) was substituted, keeping the same ABC complexes and detection technique that was provided in the Elite ABC HRP kit.

Detection was with 3,3'-diaminobenzidine (DAB) solution (Sigma-Aldrich®; #D4293) made up in distilled water (with vortexing) and the addition of the urea tablet provided, with further vortexing. The DAB solution was made up and used within 1 hour.

2.6.2.5.1 Isotype Specificity

In theory there should be minimal isotype crossover between anti-IgG and anti-IgM secondary antibodies with the IgM and IgG primary antibodies, respectively. However, with manufacturer of the Telomerase 2C4 antibody stated there is crossover, with 12% of IgM primary antibodies being detectable using an anti-IgG secondary. Using this method for staining has the potential to vary from batch to batch and the vendor could not guarantee consistency of results if an anti-IgG secondary was being relied on to detect an IgM primary antibody. Hence the decision was made to use isotype specific primary and secondary antibody combinations to overcome this potential issue.

2.6.2.6 Reducing Background Staining

Various blocking steps were used throughout the optimisation process. These included, blocking with secondary antibody host animal serum, using secondary host sera to dilute primary antibody to a working concentration, shortening primary antibody incubation times, diluting the antibodies (primary and secondary) and DAB dilutions were all explored to give the best possible staining in positive control tissues. This work was felt to be critical given that subcellular location was to be described in tumour and background tissues.

2.6.2.7 Telomerase IHC Optimisation

The optimisation processes for the Survivin IHC involved fewer steps, compared to the Telomerase antibody, confirming a higher confidence in the data generated. Regrettably there has not been an equivalent quantity of published research assessing the use of IHC to assess tissue expression of Telomerase, perhaps due to poorer quality antibodies available, which necessitated significant optimisation steps as will be discussed later.

There are fewer published works that have used IHC for Telomerase protein quantification to draw from as potential sources of antibody. In fact, there are some who question the quality, sensitivity and specificity of the antibodies that are commercially available and state that "hTERT immunohistochemistry data should be interpreted with caution," as a result (Wu *et al.*, 2006; Kim *et al.*, 2013).

However, as a key step in a rat HCC model being the nuclear translocation of Telomerase (Chen and Kong, 2010). Also, other researchers focussing on nuclear positivity when detecting Telomerase with IHC in various tumour served as a basis for our IHC interpretation of these results (Lotfi *et al.*, 2014; Saeednejad Zanjani *et al.*, 2019).

There is increasing evidence that non-tumour tissues can possess Telomerase activity with typically cited examples being testis, ovary, placenta and thymus tissues (Hiyama *et al.*, 2001). An obvious starting point in the optimisation process is the selection of both a positive control tissue, and an antibody that that detects appropriately. Small volumes of antibodies were purchased, used at 1:100 dilution, on the five different tissue types as outlined in figure 9.

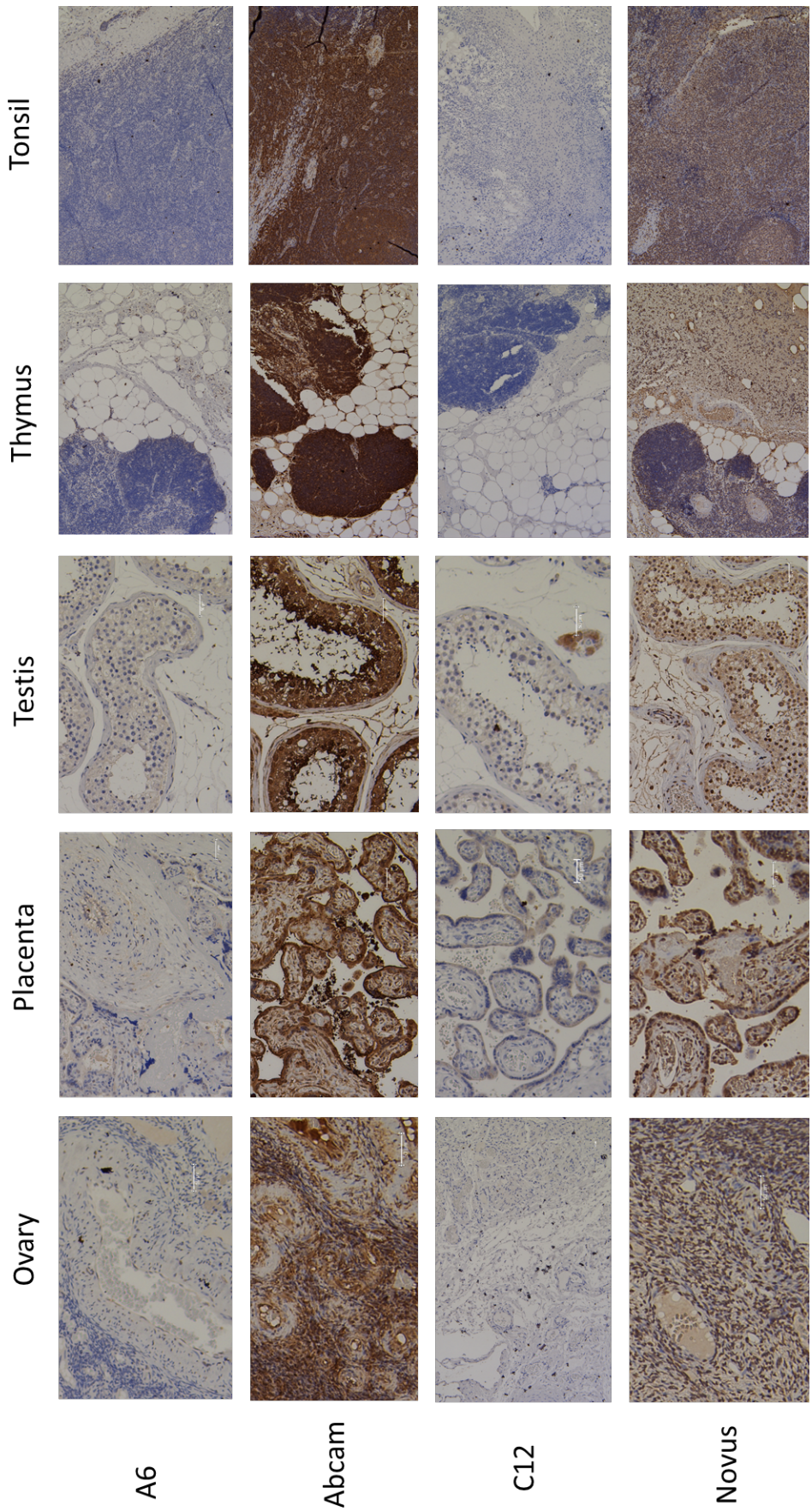


Figure 9. Four different antibodies used on five different tissue types to find a suitable positive control. Citrate antigen retrieval was used for all with antibodies used at 1:100 dilution. Imaged at 200X.

With expert review, the most consistent antibody was felt to be the Novus, with best staining in normal testis. Other, less referenced, antibodies either gave extremely vague (A6, C12) or non-specific positivity (Abcam) compared to the Novus Antibody. Further optimisation, including blocking steps, can be seen in figure 10.

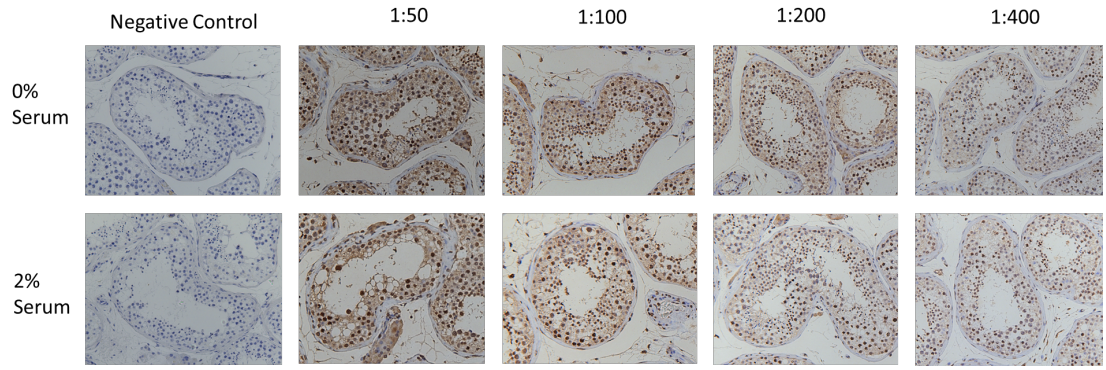


Figure 10. Goat blocking serum for diluting primary antibody.

Numerous steps were introduced to reduce the non-specific background staining from the Novus antibody. As outlined in figure 10 a 2% goat serum block was used, and the protocol was altered from overnight incubation with primary antibody (4 Celsius) to a one-hour (RT) incubation step which greatly reduced the non-specific staining.

To ensure consistency with previously published work, figure 11 demonstrates positively staining spermatids, with less intensely stained primary and secondary spermatocytes. The left panel shows a 1:400 dilution of primary antibody in 2% goat serum, incubated for one hour at RT, on normal testis with spermatocytes, spermatids and spermatozoa staining positively at 200X magnification with 100micron scale bar. Right panel shows a previously published image of human testis with staining in the spermatocytes (Hiyama et al., 2001).

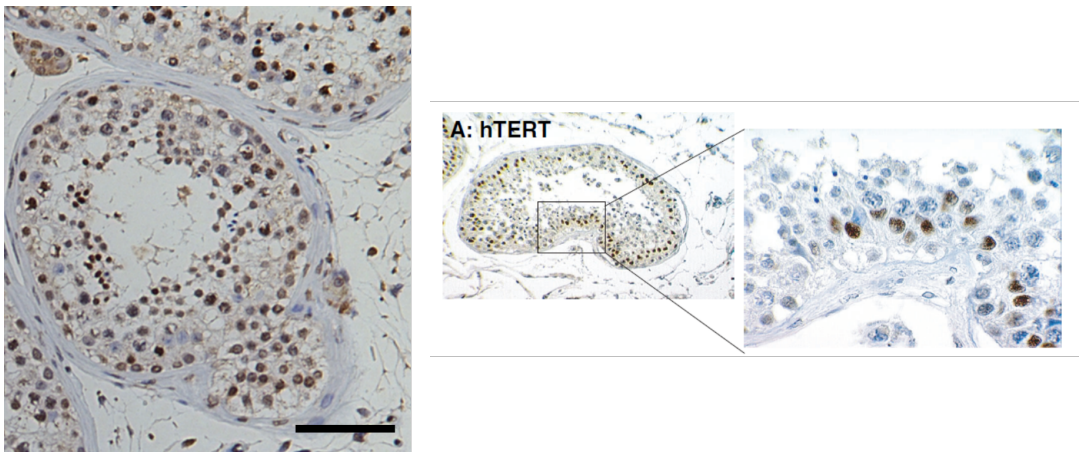


Figure 11. Telomerase expression in testicular tissues.

Therefore, the positive control tissue was decided as normal Testis, with a primary antibody diluted to 1:400 in a 2% (v/v) goat serum.

2.6.2.8 DAB Supply

Initial purchases of SIGMAFAST™ 3,3'-Diaminobenzidine tablets worked very well for detection of Survivin protein in tonsil and liver tissues. Owing to unforeseen delays in receiving delivery from the manufacturer an alternative source was required for detecting Telomerase. Repeated rounds of optimisation were necessary to ensure comparable staining with an adequate substitute.

2.6.3 Experimental Procedure

Sections were incubation at 60 °C for thirty minutes to de-waxed the slides, followed by two five-minute washes in xylene (Fisher Scientific) and two further five-minute washes in 100% ethanol (VWR®). Sections were then washed in running tap water for five minutes before blocking by submersion in 3% hydrogen peroxide (Fisher Scientific) in methanol (VWR®) for 30 minutes at RT, then washed in running water for 10 minutes. Antigen retrieval was performed by pre-treatment with either EDTA buffer (6.8 mM EDTA, 19.81 mM Tris base) pH 9.0 or citrate buffer (10 mM citric acid) pH 6.0. Sections were boiled in pre-treatment buffer for 30 minutes and then washed under running water for 10 minutes before equilibration in Tris buffered saline-Tween-20

(TBST) buffer (0.05 M Tris base, 8% NaCl, 0.045% Tween-20) pH 7.6 for five minutes. Next, sections were blocked in 1% normal horse/goat serum in TBS-T buffer for 30 minutes at RT followed by an avidin-biotin block (Vector Laboratories Ltd; #SP-2001) to block nonspecific binding of endogenous biotin consisting of a 15-minute incubation with Avidin D solution at RT, a brief one-minute wash in TBS-T and then a 15-minute incubation with Biotin solution. Finally, sections were drained of biotin solution and incubated in primary antibody diluted in TBS-T (Survivin)/ TBS-T + 2% Goat serum (Telomerase), overnight at 4 °C (Survivin) / one hour RT (Telomerase).

Sections were washed twice by immersion in TBS-T for five minutes and biotinylated secondary antibody (Vectastain® Universal Elite ABC kit; #PK-6200; Vector Laboratories Ltd) applied for 30 minutes at RT. Sections were washed twice by immersion in TBS-T for five minutes and incubated with biotinylated HRP (Vectastain® Elite ABC Reagent; Vectastain® Universal Elite ABC kit) for 30 minutes at RT according to manufacturer's protocol. Sections were again washed twice by immersion in TBST for five minutes before incubation in 3,3'-diaminobenzidine (DAB) solution (Sigma-Aldrich®; #D4293) for 5 minutes to allow detection of protein and then washed for 10 minutes under running water. Counterstaining of sections was performed with application of Mayer's haematoxylin solution (Sigma-Aldrich®; #MHS1) for two minutes and excess removed by washing under running water for 10 minutes. Finally, sections were washed twice for five minutes in 100% ethanol, followed by two five-minute washes in xylene and then mounted onto coverslips using DPX mounting agent, (Sigma-Aldrich®; #06522), applied facedown, inspected to ensure no air bubbles were obscuring the tissue, then turned face-up to allow the mounting media to set.

2.6.4 Digital Assessment of Immunohistochemistry

Whole tissue sections were scanned using the Glissando Slide Scanner (Objective Imaging) at 40X magnification and stored on encrypted hard drives (diskashur2, HDD

iStorage) to facilitate compatibility with NHS security protocols. Individual sections were assessed and sufficient data points were included to ensure the software was able to triangulate the position of tissue sections on the glass slides. Data was stored in the ".svs" format and assessed using a Lenovo ThinkCentre (Intel CORE i5 vPro 8th Gen processor) desktop computer.

The open source free-to-use software, QuPath (https://qupath.github.io) version 0.1.2, was used in the assessment and quantification of the scanned images. Version 0.1.2 is the most recent stable edition QuPath that runs glitch-free and has been used in other published articles (Bankhead *et al.*, 2017; Loughrey *et al.*, 2018). Subsequent development editions of QuPath – so called 'milestone' versions – are more up to date but may require 'bug-fixes' and caution has been advised by their creator. Hence the more stable version of the software was used. Online tutorials were extremely informative and outlined the main features of the software package (Bankhead, 2018). QuPath's positive cell detection, along with the addition of smoothed features, creates 67 different parameters for assessing individual cells in the study tissues. Random trees classifiers were used on each image, utilising the interactive nature of the software to learn the various tissue types present. As cells are classified based on morphological and environmental features representative regions of tumour, background liver, inflammatory cells, stromal tissues and necrotic tissue were used, when present, for this optimising process. Detection classifiers were created that identified various cellular subtypes that could then be assessed for DAB staining intensity.

Tumour cells were classified as being negative (-), weakly positive (+), moderately positive (++) or strongly positive (+++) based on the intensity of DAB nuclear positivity. It is important to note that the software is unable to differentiate between true nuclear staining (denoted by haematoxylin overlaid by DAB) from any artefactual/incidental haematoxylin/DAB overlay, unless trained to do so by a classifier.

If there is nonspecific staining that overlays haematoxylin, this could be erroneously interpreted as a positively stained nuclei, as was evident when using the Telomerase antibody.

The intensity of staining was determined using pre-set values (<0.2 was negative, ≥0.2 but <0.4 for +, ≥0.4 but <0.6 for ++ and ≥0.6 for +++) for Survivin quantification. For Telomerase an optimisation process was required and intensity measures of adjusted accordingly (<0.3 was negative, ≥0.3 but <0.5 for +, ≥0.5 but <0.7 for ++ and ≥0.7 for +++) as described later. Short sequences of code were written (using the 'script' function) to ensure these optimisation steps were included for each image assessed and that each object selected was appraised to ensure consistent results. The addition of "selectAnnotations();" into the code meant that when an area of tissue was highlighted for assessment, regions could be excluded if there were potential contaminants such as ink present, or a tissue fold that would give a false reading. Small areas (approximately 5000 cells) were used to ensure the accuracy of positive cell detection, and that cells were being classified appropriately prior to whole slide assessment.

Internal positive controls (predominantly inflammatory cells) were useful in ensuring the quality of the IHC undertaken, and were excluded from the analysis of staining intensity owing to a quirk in the software that means only tumour cells were assessed for this characteristic. As each individual classifier was trained to recognise inflammatory cells, the intensity of non-tumour cells is ignored by the current iteration of the QuPath algorithm. This script was then run on a whole slide basis, with care being taken to avoid the outer rim of the tissue as this could give a false positive reading (Reichling *et al.*, 2019).

The whole slide-image was assessed using the individual script of code that relied on the unique classifier that had been created for each tissue sample. This ensured the

sensitivity of tumour/background/inflammatory cell detection was tailored to each individual slide and therefore ensured maximal accuracy in this measurement.

2.6.4.1 Project Size & Classifier Clarifications

The Project is the file name for samples that are assessed together. The classifier is the tool that undertakes this assessment and can either be used across a number of samples (in a project) or can be created for each individual tissue sample being assessed.

Assessment of tissues have been made after dividing them into groups based on histological subtype and using a single classifier to detect similar cells across a number of slides in one project (Reichling *et al.*, 2019) or assessed a whole project simultaneously, having identified individual cells of interest on each slide when creating the classifier (Morriss *et al.*, 2020). A third method of assessing tissues has also been published and involves teaching the object classifier for cell subtypes on each slide (Stålhammar *et al.*, 2019). There are limited reports of the time required for these processes to be undertaken but the range cited for slide assessment varies from less than one minute (using the powerful Intel Xeon Gold 6128 CPU processor) to ten minutes per slide (K. Liu *et al.*, 2019; Morriss *et al.*, 2020).

The potential benefits of being able to teach the classifier a wide range of morphologically diverse tumour types in training and validation cohorts. This would allow a powerful computer to use machine learning to detect malignant cells based on a very large training cohort as demonstrated by the 843 training and 212 validation cases Liu et al studied when studying nasopharyngeal carcinoma (K. Liu *et al.*, 2019).

However, as the ethical stipulations associated with this project mandate that the researcher be blinded to the patient characteristics and outcomes when studying the tissues, histological subgroup analysis was not appropriate as this would have involved a complex, partial, un-blinding process that would have violated these stipulations.

2.6.4.2 Creating an Object Classifier

The process of teaching QuPath what cells to classify as tumour, inflammatory cell, stoma or background liver is intuitive and relatively simple. A visual guide is outlined in figure 12.

With the page viewed in landscape, in the top left image, a yellow box has been drawn and will be used to teach QuPath how to process of detection and assigning cell classes within it. The 'positive cell detection' tool has been used in the top right image, where haematoxylin and DAB overlay each other. Note the arrowheads in the upper left of the image – these are not tumour cells but are being detected as a 'positive' cell by the red colouring. In the bottom left picture, the previously arrow-headed stromal cells have been highlighted in a drawn on blue ring (see arrow) and assigned as 'stroma.' QuPath has now *learned* that cells appearing similar to these (non-tumour) cells are re-classified within this optimisation box. Another arrow in the centre of the bottom left image is teaching QuPath what tumour cells look like. Other shapes (both in red and blue) have been drawn into this space over similar looking (either tumour or stroma) to better educate the software.

Finally, the intensity-assessment is made (see bottom right image) across the whole tissue sample (note no box around the small number of cells as in the three other images) that gives blue (negative), yellow (+), orange (+ +) and red (+ + +) staining values. Pre-set and manually entered intensity values can be used for this step. Both the stroma and any negatively staining cells have been assigned the colour blue in this illustrative example.

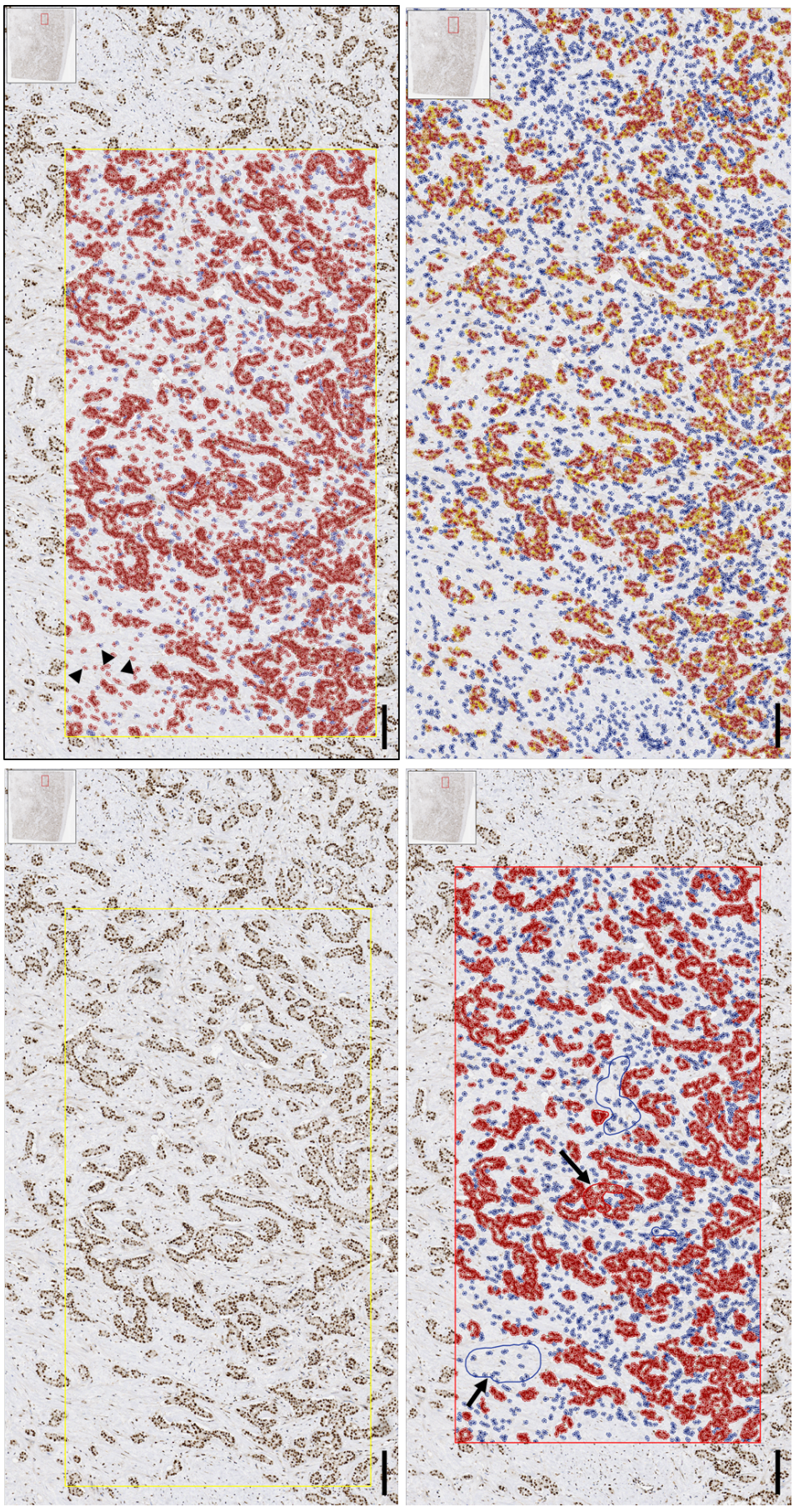


Figure 12. Creating an object classifier in QuPath.

Once the image has been processed in its entirety, the processed image is quality controlled by visual inspection and, if it appears satisfactorily accurate, QuPath is then able to report Allred and H-scores. The time taken to teach the object classifier for a new image is approximately 1-2 minutes, with the following full slide assessment taking about 4-8 minutes, depending on the size of the data file. This is comfortably within the ten minutes previous researchers have quoted for the full assessment of each sample (K. Liu *et al.*, 2019; Morriss *et al.*, 2020).

2.6.4.3 Trial Datasets

Survivin stained tissues were the first to be assessed using the QuPath software in this cohort. A consultant Histopathologist with a subspecialty interest in hepatic pathology, Dr Jemimah Denson, assessed the tissues for their intensity of staining, and the percentage of cells staining positively. Dr Denson assessed a small cohort of randomly selected samples and the respective scores compared with those reported by QuPath.

When the test cohort was assessed on QuPath and compared to Dr Denson's quantification it quickly became apparent that attempting to use a single classifier across different tumour types would result in poor quality data. Examples of these issues include the computer slowing down considerably and intermittently crashing when the project size (number of images added to one file) was larger than ten images. As, on average, ten images account for between 30GB and 40GB of data that were open and being assessed simultaneously the available computer power meant an alternative approach would be necessary.

Intracellular accumulation of lipoproteins is present in hepatocytes to varying degrees from different patients. These accumulations are associated with chronic liver dysfunction and their presence varies widely in across tissue samples. An advantage of creating individual detection classifiers means that the presence of these inclusions can

be accounted for when assigning a particular morphological feature to a tumour cell or normal hepatocyte.

After re-assessing the staining of a small cohort of slides, there was considerable agreement between the algorithm and Dr Denson's assessment. The quality of the Survivin antibody is readily apparent, and had been used in peer-reviewed publications more than 200 times, at the time of selection, including for the use of IHC on FFPE samples.

2.6.4.4 Optimising Telomerase IHC interpretation

The Survivin cohort was analysed using the pre-set values for staining intensity on QuPath. Regrettably when the same approach, using the installed values for staining intensity, was attempted in a test cohort of Telomerase IHC tissues the resulting data was inaccurate. An optimisation phase for assessing the Telomerase antibody-staining intensity was required that necessitated a range of cut off scores for to be tested and compared with Consultant Histopathologist, Dr Denson's, scores. The parameters are outlined in table 16.

Intensity Scores	**Negative**	**1+**	**2+**	**3+**
Pre-Set	<0.2	≥0.2 but <0.4	≥0.4 but <0.6	≥0.6
Alternative 1	<0.3	≥0.3 but <0.5	≥0.5 but <0.7	≥0.7
Alternative 2	<0.4	≥0.4 but <0.6	≥0.6 but <0.8	≥0.8

Table 16. Parameters used for optimising staining positivity for Telomerase IHC. Intensity measures are arbitrary units based on the optical density sum.

A small batch of ten randomly selected samples was assessed using an object classifier whose only difference was the gated values for intensity scores outlined in table 16. This meant that there was minimal sample processing (i.e., none - other than the intensity values altering) between runs to ensure the only variable that changed between assessments was the staining intensity score. These results were then compared with Dr

Denson's assessment of the tissues meaning the best fit for staining intensity was Alternative 1.

This is perhaps a reflection of the less published antibody used in the assessment of Telomerase (used in 18 previous publications, not 219 as per the Survivin antibody) or maybe even the fact that Telomerase IHC may not be as readily interpreted, or indeed vary widely between antibodies (Kim *et al.*, 2013).

2.6.4.5 Quantifying Tissue Staining

Appropriate scoring systems are required to fully describe the extent of tissue positivity (or otherwise) when assessing immunohistochemical data. To reduce whole slide image data to a simple, transferrable, interpretable format requires a scoring system, or systems, that have been designed to convey this information. There are two classical assessment methods: The Histological score (H-score) and the Allred scoring systems. Both the Allred & H-scores were initially devised to assess breast cancer tissues to differentiate between negative and strongly positive expression, and various levels in between (McCarty *et al.*, 1986; Allred *et al.*, 1998). This has helped researchers and clinicians convey degrees of tissue positivity for steroid receptor status since the late 1980s. The H-score, out of 300, is calculated by the following formula (McCarty *et al.*, 1986):

H-score (0-300) = 0*(% 0 intensity) +1*(% 1+ intensity) + 2*(% 2+ intensity) + 3*(% 3+ intensity)

H-scores have been used in non-breast cancer research studies to quantify tissue staining metastatic liver disease from uveal melanoma and metachronous colorectal cancers (Tamagawa *et al.*, 2013; Gardner *et al.*, 2014). The Allred score incorporates both the proportion of cells (proportion score, PS) and the intensity of staining (intensity score, IS) as outlined from Allred's original paper studying breast cancer receptor staining in figure 13, (Allred *et al.*, 1998). The Allred score = PS + IS.

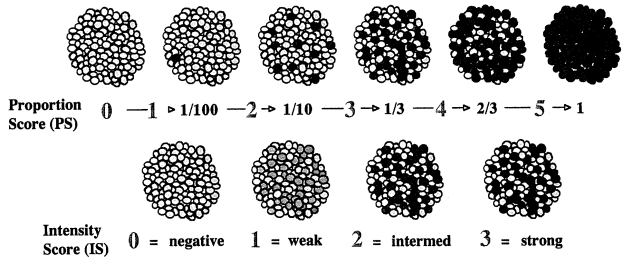


Figure 13. Visual representation of the proportion score (PS) and the Intensity score (IS) giving the combined Allred Score.

Proportion scores of 0 is for no staining, a score of 1 is for up to 1% of cells being positive, for between 1-10% of cells (score 2), 10.1-33.3% (score 3), 33.4-66.6% (score 4) and 66.7-100% (score 5). The intensity of staining requires finite values to be interpreted (which QuPath accounts for) with values of 0, 1, 2 and 3 depending on how intensely stained the cells are, to give an Allred score from 0-8. This method has been used to study steatohepatitis in hyperlipidaemia, as well as the epidermal growth factor receptor status in colorectal cancer research (Rokita *et al.*, 2013; Sturzeneker *et al.*, 2019).

The Allred score was devised to assess Oestrogen receptor positivity in breast cancer and has been used in conjunction with Progesterone receptor status since the 1990s (Rhodes *et al.*, 2020). Breast tumours are considered negative with an Allred score of 2, or lower, and have been stratified as low-expressing (Allred score 3-5) and high expressing scores (Allred 6-8) to further characterise both the extent and intensity of protein level (Campbell *et al.*, 2016). In one case series 27% of breast cancers had an Allred score of 0 and 66% a score of 8, indicating the non-normal distribution of

staining intensity as shown in figure 14, (Rhodes *et al.*, 2020).

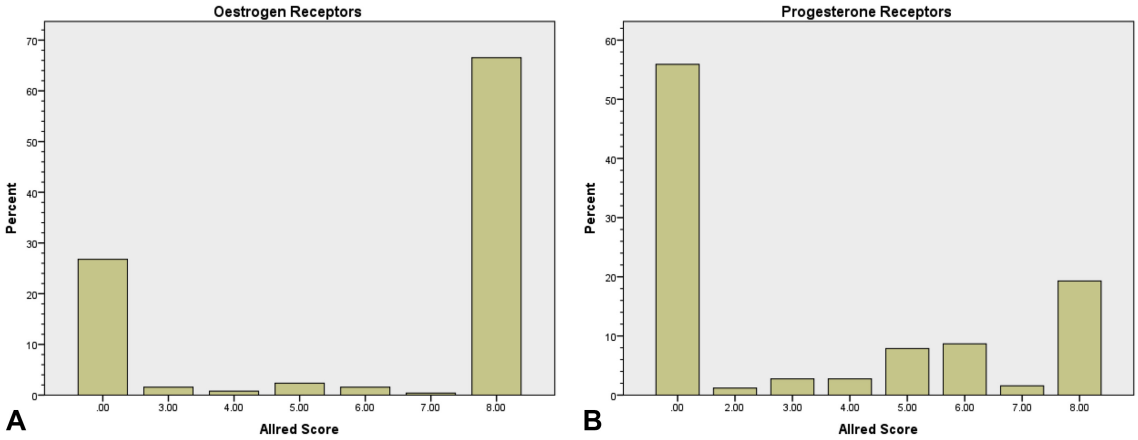


Figure 14. Allred scores for Oestrogen and Progesterone receptors in Breast cancer.

These scores were manually assessed and highlight the near-binary expression of Oestrogen receptors in this cohort of breast cancer specimens. There is clearly the potential for digital pathology to undertake some of this semi-quantification, especially when assessing a normal/near-normal distribution of protein expression. If Allred scores are normally distributed, dividing up the samples in no, low and high expression could yield greater accuracy in results. Figure 14 also gives a good representation of the positively staining (weak through to strong expression) found when semi-quantifying IHC.

As significant numbers of cells are counted, and assessed, in QuPath, both scores will be used in the subsequent analysis to compare results. There is also precedent for sub-categorising the intensity of tumour-expressing protein levels when prognosticating tumours with higher expression of Oestrogen or Progesterone (Campbell *et al.*, 2016). There has also been a phase II/III trial in breast cancer patients using the cut off values of 6-8 for 'ER rich' tumours, and tailoring their therapy accordingly, (Ellis *et al.*, 2010).

2.7 ELISA

Biobanked serum samples were thawed and used to in conjunction with commercially available enzymes linked immunosorbent assay (ELISA) kits.

2.7.1 Survivin ELISA

Serum Survivin levels were tested using the Human Survivin Quantikine® kit (R& D Systems®) using the manufacturers protocol as detailed below. Owing to the uncertain effect serum would have on the ELISA, experiments were repeated with both the provided diluent, and then with a 10% (v/v) healthy volunteer serum as the diluent for the standards. All samples, including controls, were run in duplicate.

2.7.1.1 Preparation of Standards

The Quantikine® standard was reconstituted from the lyophilised form with addition of 900µL of deionised water, to give a stock concentration of 20,000 pg/mL, and allowed to completely reconstitute for 15 minutes. Subsequently, 100µL of this stock concentrate was added to 900µL of calibrator diluent, RD6-47, to give a high-standard concentration of 2000pg/mL, and mixed by pipetting. Serial dilutions were then made using equal parts calibrator diluent (500µL) and the mixed preceding concentration, as outlined in table 17. The calibrator diluent was used as the zero standard.

Vial	Volume of Diluent (µL)	Volume (µL) & source of standard	Final Concentration (pg/mL)
A	900	100, Stock concentrate	2000
B	500	500, vial A	1000
C	500	500, vial B	500
D	500	500, vial C	250
E	500	500, vial D	125
F	500	500, vial E	62.5
G	500	500, vial F	31.3
H	1000	N/A	0

Table 17. ELISA reagents using the Survivin Quantikine® kit (R& D Systems®).

For the repeat experiment to ascertain the effects, if any, of serum on the assay, the standards were diluted in 10% (v/v) healthy volunteer serum in the provided calibrator diluent. Reconstitution of the lyophilised standard was undertaken with deionised water only. The low standard (vial H) was 10% (v/v) healthy volunteer serum only.

The Quantikine Immunoassay Control Group 8 (catalogue number QC23, R& D Systems®) was used for an assessment of the assay function. Controls are present in low (143 – 360 pg/ml), medium (385 – 1015pg/ml) and high (834 – 1815 pg/ml) concentrations having been prepared in diluted porcine serum and subsequently lyophilised. The control samples contain numerous recombinant human cytokines in each vial.

2.7.1.2 Experimental Procedure

Microplates were removed from storage at -20 Celsius and left to equilibrate to RT. Room temperature assay diluent (RD1-9) was added to each well (100 µL) with equal volumes of standard, control or sample per well, to give a total volume of 200µL in each well. An adhesive strip was then used to cover the wells which were then incubated at RT for two hours on a horizontal orbital microplate shaker (Thomas Scientific, 0.12" orbit at 500 ± 50rpm). Well contents were then aspirated and washed with 400 µL (1x concentration) wash buffer for a total of four wash cycles using a multichannel pipette. Residual liquid was removed with inversion between steps, with blotting against clean paper towel after the last wash step.

Human Survivin conjugate (200 µL) was then added to each well, covered with a new adhesive strip and incubated at RT for two hours on a horizontal orbital microplate shaker (Thomas Scientific, 0.12" orbit at 500 ± 50rpm). Aspiration and wash cycles (x4) were repeated. Substrate solution (200µL) was added to each well and the samples were covered and incubated at RT on the benchtop whilst being protected from light, for thirty minutes. Following this 50 µL of Stop solution was added to each well allowing a homogenous change in colour from blue to straw-yellow. Optical density (OD) was then measured within 30 minutes at both 570 nm and 540 nm with the recorded reading corrected for the 570 nm wavelength (540 nm – 570 nm) to correct for optical imperfections in the plate.

For calculating the relative differences in absorbance between wells, the 540 nm-570nm readings were used and each reading had subtracted the zero-standard (0 pg/mL) reading to demonstrate relative differences in absorbance.

2.7.1.3 Calibration Curves

Samples were run in duplicate and calibration curves were calculated for each ELISA run. These calibration curves can be seen in figures 15 & 16, depending on the diluent used. Error bars demonstrate the standard deviation.

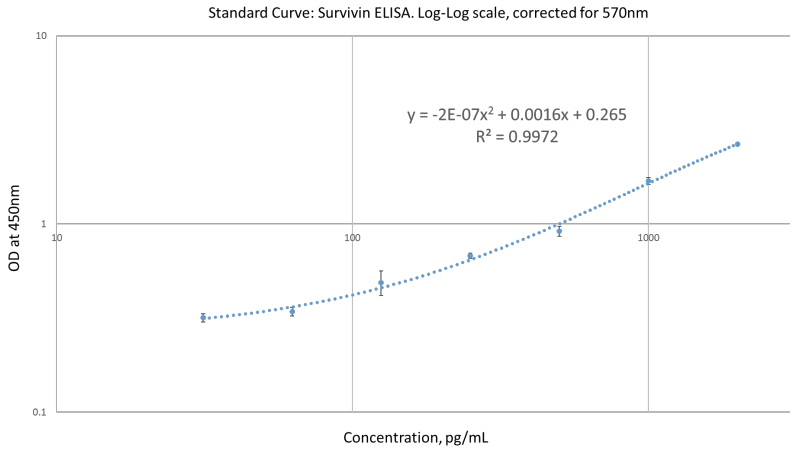


Figure 15. Standard curve used to correlate optical density (OD) with concentration using the calibrator diluent.

To account for other, serum, effects these experiments were repeated, as shown in figure 16.

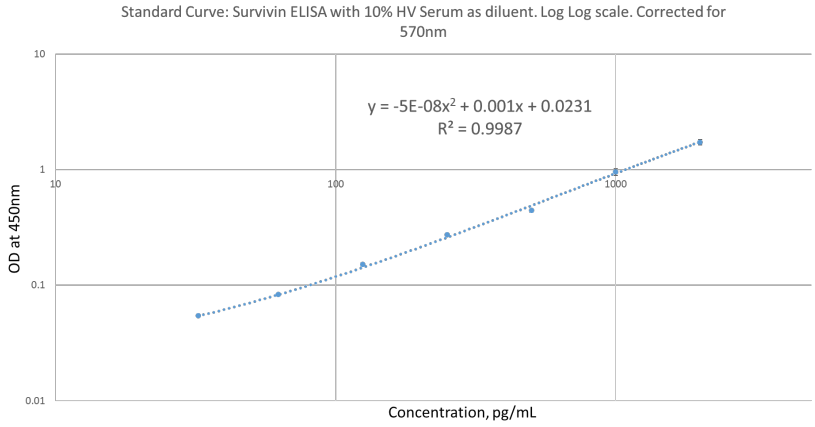


Figure 16. Standard curve used to correlate optical density (OD) with concentration using 10% (v/v) healthy volunteer (HV) serum in calibrator diluent.

2.7.2 Telomerase ELISA

Serum hTERT was detected using the Human Telomerase Reverse Transcriptase (TERT) ELISA Kit (abx251454, abbexa®). Samples and standards were run in duplicate and the effect serum had on the assay was ascertained by running the experiment with calibrator diluent (as provided by the manufacturer) and in 10% (v/v) healthy volunteer serum.

2.7.2.1 Preparation of Standards

To ensure all of the lyophilised powder was used, the dry powder was centrifuged briefly to ensure there were no residues in the cap of the vial. To the lyophilised powder, 1 mL of Sample/Standard diluent was added and mixed by pipetting and then allowed to sit for 15 minutes at RT. Care was taken to avoid mixing by vortexing as this would destabilise the protein. Once reconstituted, each standard was used immediately and then discarded to ensure optimal accuracy in measurement. Subsequent serial dilutions of the standards are outlined in table 18.

Vial	Volume (µL) of additional Sample/Standard Diluent	Volume (µL) & source of standard	Final Concentration (ng/mL)
A	N/A	1000, Standard.	10
B	500	500, vial A	5
C	500	500, vial B	2.5
D	500	500, vial C	1.25
E	500	500, vial D	0.63
F	500	500, vial E	0.31
G	500	500, vial F	0.16
H	500	N/A	0

Table 18. Standards prepared from the ELISA Kit (abx251454, abbexa®).

For the repeat experiment to ascertain the effects, if any, of serum on the assay, the standards were diluted in 10% (v/v) healthy volunteer serum in the provided sample/standard diluent. The low standard (vial H) was 10% (v/v) healthy volunteer serum only.

2.7.2.2 Experimental Procedure

All reagents and kit components were equilibrated to room temperature before use. Into each well, 100 µL of standard, sample or zero control was added, covered with adhesive strip and incubated at 37 Celsius for 90 minutes. Following this incubation step, the liquid contents was discarded and (without a wash step) 100 µL of the biotin conjugated antibody was added to each well. A new adhesive coverslip was added and then incubated at 37 Celsius for a further 60 minutes.

The well contents were discarded and three washes with 1X wash buffer were undertaken using a multi-channel pipette, with a 90 second soaking period for each wash step. Well contents were completely removed (with inversion) after each stage and after the final wash step the plate was inverted and blotted against clean absorbent paper towels. 100 µL of HRP working solution was then added to each well, covered with new adhesive plastic and incubated at 37 Celsius for 30 minutes.

The well contents were discarded and five wash steps, as described above, was undertaken. TMB substrate (90 µL) was added to each well, covered and incubated at 37 Celsius, in dark conditions, for 20 minutes. Stop solution (50 µL) was then added to each well with a concurrent colour change to a homogenous straw-yellow. Absorbance measurements were undertaken at 450 nm immediately.

For calculation of the relative absorbance, the zero-standard (0 ng/mL) reading was subtracted from the recorded reading from each well, at 450 nm.

2.7.2.3 Calibration Curves

Samples were run in duplicate and mean values were taken, as can be seen in figures 17 & 18.

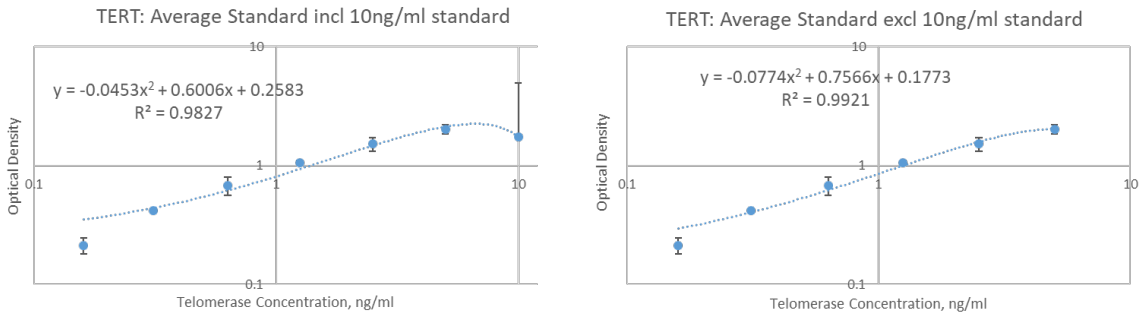


Figure 17. Standard curves used to correlate OD with concentration using only calibrator diluent with the Telomerase ELISA Kit (abx251454, Abbexa

Standard curves for samples run in duplicate using provided diluent (not 10% HV serum) are shown in figure 17. The OD reading for the 10 ng/ml high standard was lower than expected, so both calibration curves (one including the 10ng/ml reading and one excluding it) are shown.

When the calibration curve was run in 10% HV serum, the results were disappointing, as can be seen in figure 18.

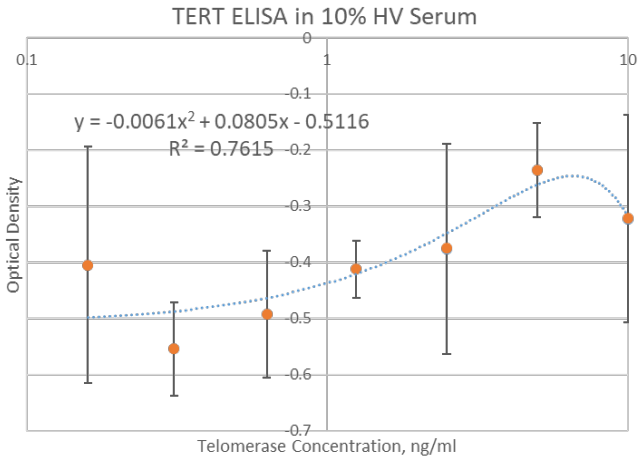


Figure 18. The standard curve used to correlate OD with concentration using 10% HV serum as calibrator diluent from the Telomerase ELISA Kit (abx251454, Abbexa.

As can be seen from the calibration curve in figure 18, there is no clear relationship between telomerase concentration and optical density when the standards are diluted in 10% healthy volunteer serum. Some OD values were negative, and so a log-log graph cannot be calculated.

2.8 Exosome Work

Exosomes were extracted from biobanked serum samples using a commercially available kit that exploits the precipitation method as has been reported previously (Gutkin *et al.*, 2016; Goldvaser *et al.*, 2017). Choosing the precipitation method over ultracentrifugation was prudent as processing could be undertaken using routine bench top equipment and currently has the maximal exosomal yield when compared to other methods of retrieval (Patel *et al.*, 2019).

2.8.1 Experimental Procedure

2.8.1.1 Exosome Isolation

The Total Exosome Isolation Reagent, from Serum (Invitrogen, catalogue number 4478360) was used and the procedure followed was, briefly, outlined below. Serum samples were removed from the -80 Celsius freezer defrosted and placed on ice. Serum samples were centrifuged at 2000 x g for 30 minutes at 4 Celsius to remove cells and debris. The supernatant of clarified serum was transferred to an RNAse/DNAse free Eppendorf tube, taking care to not disturb the cellular pellet, and placed on ice. Workable volumes of clarified media (250 µL) had 0.2 volume equivalents (50 µL) of Total Exosome Isolation reagent added to them and were mixed vigorously by pipetting until a cloudy, homogenous, solution was present. The reaction mixture was then stored at 4 Celsius for 30 minutes. Following this step, the reaction mixture was then centrifuged at 10000 x g for 10 minutes at room temperature. The supernatant was discarded to leave a yellow/orange pellet of exosomes in the bottom of the Eppendorf. A single wash step involved the addition of 1x PBS (Thermofisher) and then carefully removing the PBS whilst the exosomal pellet remained at the bottom of the Eppendorf tube. The Exosomal pellet was resuspended in 500 µL QIAzol (Qiagen) with care taken to work in a fume hood and work with filtered tips. The resuspension phase can take up

to 15 minutes with larger exosomal pellets and involves mixing extensively by pipetting.

2.8.1.2 RNA Extraction

2.8.1.2.1 Exosomal RNA

Chloroform (Sigma Aldrich), 100µL, was added to the samples when under the fume hood with a p1000 filtered pipette and mixed by shaking manually for 15 seconds. Samples were then centrifuged at 12000 x g for 15 minutes at 4 Celsius. Following centrifugation, the clear upper phase was transferred to a new RNAse/DNAse free Eppendorf tube for RNA extraction. Care was taken to not aspirate the white middle layer (protein) or touch the sides of the Eppendorf tube whilst pipetting to minimise contamination at this stage. PCR grade Isopropanol (Sigma Aldrich) (250µL) was added to the samples and mixed by flicking the Eppendorf tubes. The samples were then stored overnight at -20 Celsius to facilitate RNA precipitation.

The following day the samples were removed from -20 Celsius and centrifuged at 12000 x g for 10 minutes at 4 Celsius. The supernatant was removed, discarded, and then 500µL of 75% molecular grade ethanol (Sigma Aldrich) was added to the stable RNA pellet. Samples were then centrifuged at 7500 x g for 5 minutes (4 Celsius) and then the supernatant was carefully removed and discarded. At this stage the RNA pellet was less stable so some of the dilute ethanol was left in the Eppendorf and left to air-dry on the bench top.

The RNA pellet was then resuspended in 40µL nuclease free molecular grade water (who/where) then incubated at 56 Celsius on a heatblock (Dri-Block DB 2D, Techne) for 10 minutes. Following this step, the RNA could be quantified and quality assessed using a NanoDrop™ (2000 spectrophotometer Thermo Scientific™) comparing nucleic

acid contents to the nuclease free water used for the final dilution step. Extracted RNA was either used immediately or stored at -80 Celsius prior to further experimentation.

2.8.1.2.2 Cell Lines

The Huh7.5 cell line was used as a calibrator for qPCR. The TRIzol extraction method was used for RNA extraction, with spin-column purification using Direc-zol RNA Miniprep (Zymo Research, catalogue number R2050). The experimental procedure is outlined briefly below.

Growth media was removed and 0.3mL of TRIzol™ reagent (Invitrogen, catalogue number 15596026) was added per $1x10^6$ cells directly into the culture dish, with homogenisation by pipetting. Samples were then incubated for five minutes at RT. Equal volumes (0.3mL per $1x10^6$ cells) of ethanol (purity > 99.8%, Honeywell) was added to the lysed sample. The liquid was then transferred to a Zymo-Spin IICR Column (Zymogen) in a new DNase/ RNase free collection tube. The sample was centrifuged (10000 x g, 30 seconds, RT) and the flow-through discarded.

DNase 1 treatment was undertaken and the reagents were mixed in a separate Eppendrof tube: 6µL of DNase 1 to 75µL of DNA digestion buffer, and mixing by gentle inversion. The addition of RNA Wash Buffer (400µL) into the spin column, with centrifugation (10000 x g, 30 seconds, RT), discarding the flow through and then the addition of the DNase 1 mix. Incubation in the spin column for 15 minutes at RT.

Then 400µL of Direct-zol RNA PreWash was added to the column, centrifuged (10000 x g, 30 seconds, RT), and the flow through was discarded. The Pre-Wash step was repeated once more. Washing the RNA by addition of 700µL RNA Wash Buffer to the column and centrifuged (10000 x g, 1 minute, RT), followed by transfer of the column into a sterilised DNase/ RNase-free Eppendorf. The RNA was eluted with the addition

of 50µL of DNase/ RNase-free water, followed by centrifugation (10000 x g, 30 seconds, RT).

The RNA was assessed using the NanoDrop™ 2000 spectrophotometer (Thermo Scientific™) to assess both the quality (260/280 ratio) and quantity (concentration, in ng/µL) of extracted RNA. Only RNA with a 260/280 ratio >1.8 was used for subsequent experiments. Aliquots were stored at -80 Celsius.

2.8.1.3 Reverse Transcription

The reverse transcription of RNA to cDNA was done using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor (Catalogue number 4374967) as outlined below.

Kit components were thawed on ice and a 2X mastermix was created as outlined in table 19, making n+1 aliquots for n samples.

Component	Volume per reaction
10X Reverse Transcription Buffer	2.0 µL
25X dNTP Mix	0.8 µL
10X Reverse Transcription Random Primers	2.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL
RNAse Inhibitor	0.5 µL
Nuclease Free Water	3.7 µL
Total Volume	**10.0 µL**

Table 19. Reverse transcription reaction volumes for the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor.

The 2X mastermix was mixed with gentle vortexing and centrifugation. The final reaction was assembled with the addition of 1 µg of RNA added to 10 µL of the 2X mastermix and additional nuclease free water to make a final reaction volume of 20 µL in nuclease free Eppendorfs. The blank, no template control, reaction mix had 10 µL of nuclease free water added to 10 µL of the 2X mastermix to ensure no contamination had occurred that would be alter the experimental findings.

After briefly centrifuging the reaction mixtures to eliminate air bubbles and ensure reaction mixtures were in the bottom of the Eppendorfs, they were placed on ice. The Thermal Cycler (Labtech GS4 G-Storm, Gene Technologies) was set with the following steps: table 20.

Settings	Step1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 minutes	120 minutes	5 minutes	∞

Table 20. Reverse Transcription reaction conditions for Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor.

Once the reaction was complete samples were stored at 4 Celsius until processed for the qPCR.

2.8.1.4 qPCR

Quantitative PCR was undertaken using the Applied Biosystems™ TaqMan™ Fast Advanced Master Mix (catalogue number 4444557) and TaqMan™ gene expression assay probes for Survivin (BIRC5, Assay ID Hs00977611_g1, catalogue number 4331182), Telomerase (TERT, Assay ID Hs00972650_m1, catalogue number 4331182) and the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1, Assay ID Hs02800695_m1, catalogue number 4331182). Each of the assay probes contained the FAM™ reporter dye labelled TaqMan™ minor groove binder (MGB) moiety and were purchases in 250 reaction quantities. The specific assays were selected because they targeted nucleic acids that spanned more than one exon with respective amplicon lengths of 113 base pairs (BIRC5), 57 base pairs (TERT) and 82 base pairs (HPRT1).

The experimental reagents were used in the following quantities as outlined in table 21. Volumes for n+1 reactions were made up to account for pipetting error.

Component	Volume per reaction (µL)	Final Concentration
Mastermix	10.0	1X
Assay Probes	1.0	1X
Nuclease-free water	7.0	-
cDNA	2.0	-
Total volume	20 µL	-

Table 21. TaqMan qPCR reaction mix.

With the reaction conditions in a Lightcycler 480 (Roche) thermocycler as outlined in table 22.

Step	Temperature (Celsius)	Time	Ramp Rate
Hotstart	95	2 min	4.4
45 cycles of 1 & 2.			
1. Denature	95	15 sec	4.4
2. Annealing	60	1 min	2.2
Followed by a single cooling step			
Cooling	40	30 sec	2.2

Table 22. Reaction conditions, as outlined by for use with the TaqMan and custom TaqMan Gene Expression Assays.

The sealed 96 well plates were then frozen for subsequent analysis, should it be required.

3 Promoter Sequencing Results.

3.1 Introduction

As detailed in chapter 2, DNA from archived samples was often of poor-quality samples and difficult to sequence, often requiring multiple cycles of PCR reaction, gel analysis, purification of PCR products and finally sending samples off for Sanger sequencing. DNA extracted from very early samples (DNA 1 – 6) were of such poor quality that repeat processing was required, so these study numbers do not feature in any examples as the tissues were re-anonymised when extraction and processing of DNA was undertaken. Visits to the Derriford Combined Laboratories (DCL) proved very helpful as a similar technique (DNA extraction from FFPE samples and PCR amplification) is used clinically for the assessment of methylation status of brain tumours for the O(6)-methylguanine-DNA methyltransferase (MGMT) DNA repair gene and assessing epidermal growth factor receptor (EGFR) mutations in lung cancer (Bethune *et al.*, 2010; Thon, Kreth and Kreth, 2013).

3.1.1 Survivin PCR Optimisation

Initial sequencing work was undertaken targeting the promoter region upstream of the Survivin transcription start site. Some considerable optimisation was required to ensure an appropriate PCR product was achieved consistently.

3.1.1.1 Primer Selections

Initial reactions were undertaken using previously published primer pairs/reaction conditions to explore potential combinations for use in the whole cohort. In figure 19 DNA extracted from FFPE colorectal carcinomas (samples 16c and 17a) were run on a 2% gel alongside a 100base pair ladder.

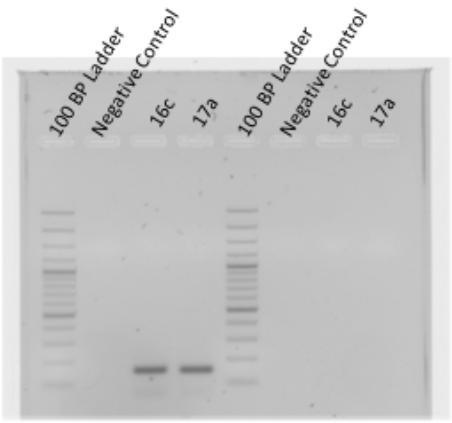


Figure 19. A 2% Agarose gel for the PCR products in test samples 16c and 17a.

Figure 19 highlights bands that are present at roughly halfway between 100 and 200 base pairs when the SurvF1R1 primer pair (annealed at 57 Celsius, 151bp PCR product) was used, confirmed with DNA from two separate tumour samples. The smaller PCR product (128 base pairs) was non-detectable in both reactions, indicating a failed experiment when annealed at 55 Celsius.

Alternative PCR primer pairs were also trialled as outlined in figure 20. As figure 20 demonstrates, there were inconsistent results with the larger (329bp) PCR product, with some reproducibility (lower half) with some primer-dimer in the negative control lane.

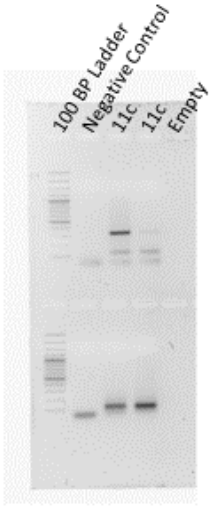


Figure 20. A 2% Agarose gel for the PCR products using DNA from sample 11c.

Expected PCR products for the primer pairs known as Han-625 (125 base pairs) and Han-31 (329 base pairs) when run in duplicate should have yielded reproducible results

when annealed at 57 Celsius (Han *et al.*, 2009). In the top panel, there is primer-dimer (present faintly in the negative control lane) and only one of two repeats have given an expected PCR product size, with a number of other non-specific bands also present. In the lower part of figure 20, there is also primer dimer present, but more consistent bands, if a little fuzzy, are present in both lanes. The inconsistent and non-specific nature of the results shown in figure 20 indicated that perhaps the best candidate for further use was the SurvF1R1 primer pair.

Sequences of fewer than 150 base pairs have not been recommended for Sanger sequencing by the LGC Genomics, meaning primer pairs that gave sequences shorter than this were potentially viable but preference would be given to a larger PCR product if this was technically feasible to produce this. Fortunately, SurvF1R1 gave a sequence of 151bp in size and the potential to further increase the PCR product size using universal primers, as discussed below.

3.1.1.2 Gradient PCR & Enzyme Selection

Initial experiments used polymerases that were available within the lab group (Dreamtaq) failed to register consistent results. Small aliquots of Accuprime PFx and Q5 polymerases were trialled and gave promising initial results.

Gradient PCR reactions facilitate a relatively rapid optimisation process for defining the best annealing temperature for PCR reactions. To maximise the potential primer combinations available to use for reactions, gradient PCR reactions were also undertaken with other promising primer-pairs. Given the Han-31 primer pair gave a 329 base pair product size, this would have made sequencing easier if it worked sufficiently well. As can be seen in figure 21 the desired PCR product size of 329 base pairs has not been achieved and there is also evidence of primer-dimer – as shown by the <100bp band in the negative (no template) control sample.

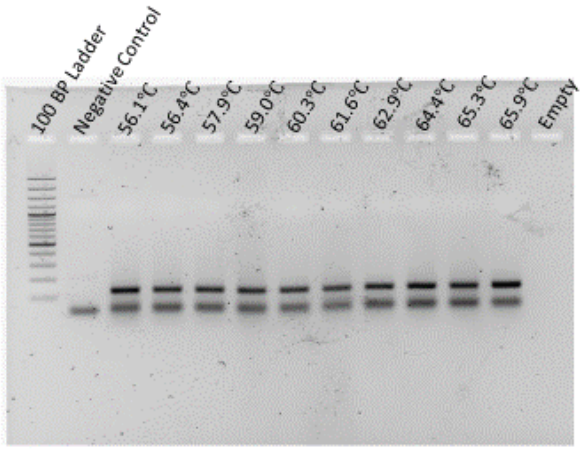


Figure 21. A 2% Agarose gel from a gradient PCR reaction using the 'Han -31' primers and the Accuprime polymerase.

Figure 21 fails to replicate previous successful PCR reactions, despite annealing over a wide range of temperatures. Given that previous groups have struggled to reproduce PCR reactions for targets >200 base pairs, it was felt the Survivin-31 F1R1 primer pair (yielding a 151 base pair PCR product size) should be used for subsequent experiments.

There were encouraging results from an alternative polymerase, Q5, and one of the initial gels is shown in figure 22.

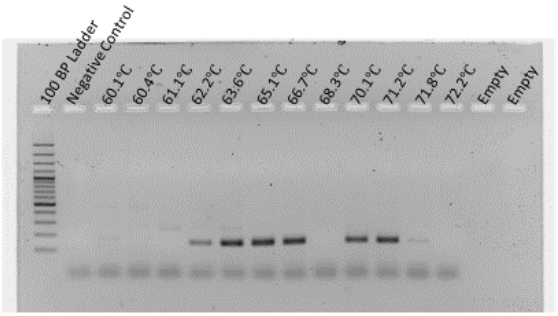


Figure 22. A 2% Agarose gel from a gradient PCR using the 'Surv F1R1' primers and the Q5 polymerase.

Regrettably when this experiment was repeated, there was no reproducibility of the PCR products.

Accuprime PFx polymerase was found to give the best results when annealing at 62.1°C, as shown in figure 23 so was ultimately adopted as the enzyme of choice for PCR reactions sequencing the Survivin promoter region.

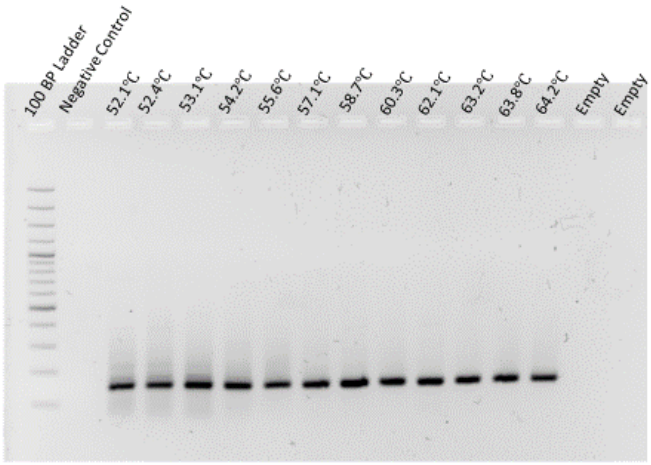
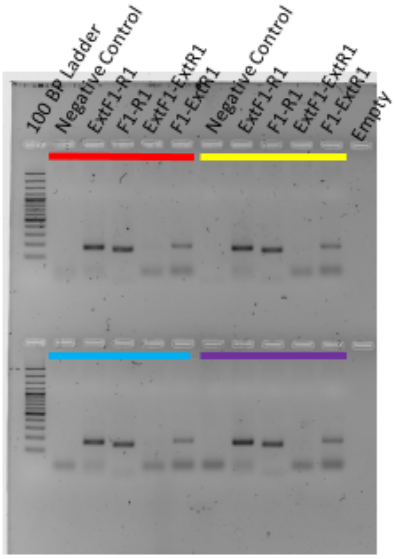


Figure 23. A 2% Agarose gel from a gradient PCR using the 'Surv F1R1' primers and the Accuprime Pfx polymerase.

There is some non-specific smear-artefact in the bands observed in figure 23 with the exception of lane 11 (annealing at 62.1 Celsius) and as such, this temperature step was selected for use with the SurvF1R1 primers, and the Accuprime Pfx polymerase.

3.1.1.3 Universal Primers

In an attempt to increase the read length of the PCR product a universal primer was added on to the 5' end of the forward primer (Kelley and Quackenbush, 1999). A number of hybrid primers were created with universal primer added to the 5' end of either the forward or reverse primer, resulting in a larger PCR product that would theoretically neatly navigate this issue, figure 24 and table 23.



Primer Combination	Predicted PCR product size (Base pairs)
ExtF1-R1	169
F1-R1	151
ExtF1-ExtR1	187
F1-ExtR1	169

Figure 24. Left side: A 2% Agarose gel with annealing temperatures as follows: Red (61.1°C), Yellow (61.9°C), Blue (63.1°C) and Purple (64.0°C). The layout outlined in the top half of the panel is replicated in the bottom half. Ext denotes the extended primer with F1 and R1 being the unaltered originals: Ext-R1 is the extended version of the R1 primer, Ext-F1 is the extended version of the F1 primer. Notably the ExtF1-ExtR1 combination failed to produce a PCR product at every annealing temperature tested.

Table 23. Predicted PCR product sizes of various primer pairs, including the additional universal primers.

The universal primers are each 18 base pairs long, giving the predicted PCR product sizes outlined in table 23 and the gel from PCR reactions in figure 24. There was some success in creating a larger PCR product using both the extendedF1-R1 (ExtF1-R1) and F1-extended R1 (F1-ExtR1). Regrettably the quantities of PCR product created in these reactions were too low (i.e., below the 100ng minimum required) to allow for Sanger sequencing.

3.1.2 Telomerase Optimisation

Experience gained from optimising PCR reactions for the Survivin promoter was transferred to the optimisation for the Telomerase promoter region. This streamlined the optimisation process as fewer primer pairs and enzymes were trialled as a PCR product of around 200 bases was the desired goal.

3.1.2.1 Primer Selection

Recently published papers used predominantly two pairs of primers when undertaking Sanger Sequencing of higher quality DNA (from frozen tissue or extracted from whole blood) and these were purchased for experiments (Nault *et al.*, 2013; Huang *et al.*, 2015; Purkait *et al.*, 2016). Primer pairs called 'short TERT' and 'TERT F3R3' yielding 163 and 190 base pair sized PCR products respectively.

3.1.2.2 Gradient PCR and Enzyme Selection

As both the Accuprime and Q5 polymerases were readily accessible these combinations were trialled, as outlined in figure 25.

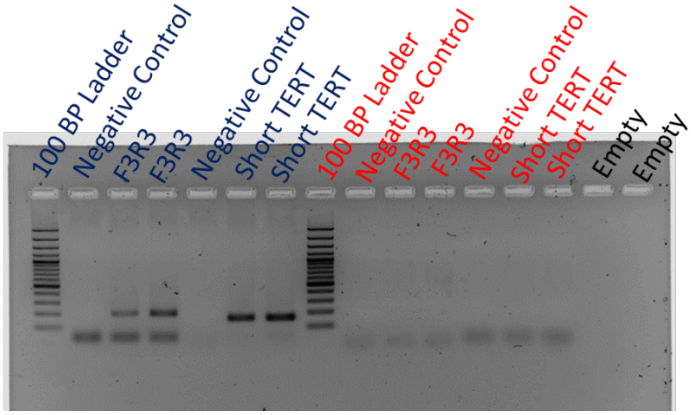


Figure 25. A 2% Agarose from PCR using Accuprime Pfx & Q5 polymerases.

However, these early results were beset with problems of a lack of reproducibility. Thus, the literature was consulted for an appropriate enzyme that had previously been used with these primer pairs. Both Chan et al and Huang and colleagues used KAPA 2G DNA Polymerase to great effect, so an aliquot of this was purchased for

experimentation (Chan *et al.*, 2015; Huang *et al.*, 2015). Gradient PCR reactions using both primer pairs can be seen in figure 26.

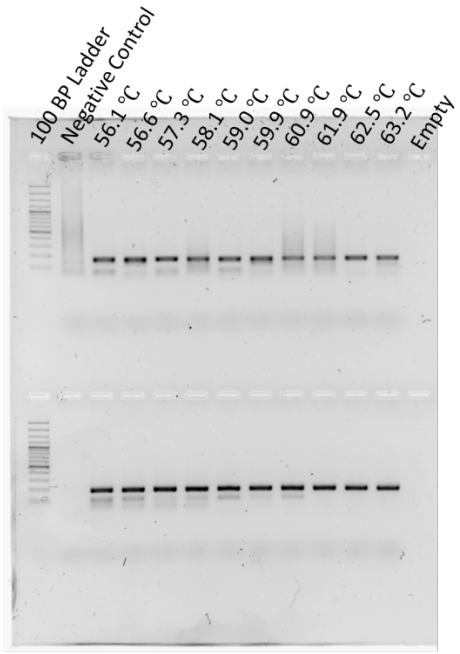


Figure 26. A 2% Agarose Gel of gradient PCR comparing primer pairs and the KAPA 2G polymerase.

Nonespecific PCR products and smearing was noted using the Short Tert primer pair (top panel). Fewer problems were observed with the F3R3 primers (lower panel) and annealing at 60°C gave a consistently good PCR product. A more focused gradient PCR reaction was undertaken and can be seen in figure 27.

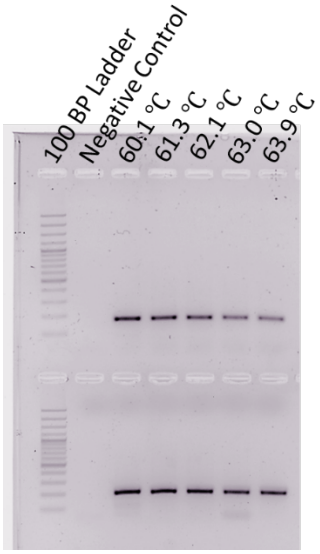


Figure 27. An abbreviated 2% Agarose Gel of a focused gradient PCR comparing primer pairs using the KAPA 2G polymerase.

The strongest band was consistently found when combining the Tert F3R3 primer pair (bottom panel) and annealing at 60.1 Celsius, giving a PCR product size of just under 200 base pairs in size. Hence reaction conditions for sequencing were set using 60 Celsius, F3R3 primers and the KAPA 2G polymerase.

3.1.3 Results from MacVector

Once the reaction conditions were determined, large-scale processing was undertaken to ensure DNA from all samples were sequenced. Once the reactions had been undertaken, gels run, PCR product purified, a specific mass of PCR product, in addition to either a forward or reverse primer, and sent for Sanger sequencing there was a repository of sequence data available for interpreting. MacVector software was used to analyse files in the ".ab1" format.

3.1.3.1 Survivin Promoter

3.1.3.1.1 Quality Assurance

Visual inspection of chromatograms was necessary to ascertain the quality, or otherwise, of the sequence data generated. Examples of both high and low-quality sequence data can be seen in figure 8.

To ensure the sequences were accurate enough for further assessment, the BLAST search function of MacVector was used which facilitated the comparison of the 'Query' (PCR product) with the NCBI database, as shown in figure 28.

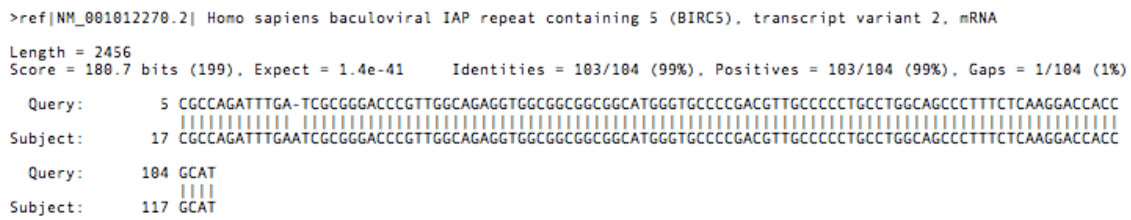


Figure 28. Output data from MacVector software comparing the PCR product 'Query' to the NCBI reference database 'Subject'. The single error is during an AA base repeat otherwise giving 103/104 bases in sequence (>99% accurate) that is recognised as homologous to Human Survivin (gene: BIRC5).

Each chromatogram was visually inspected to ensure further analysis was appropriate. A small number of initial sequences were fully assessed using the BLAST assessment tool and have been populated to give the table 24.

Sample	PCR Product Size	Sequence Orientation	Ref Seq Result
F7a	103	Forward	>99% (103 of 104)
R7a	101	Reverse	99% (99 of 100)
F7b	106	Forward	>98% (102 of 104)
R7b	103	Reverse	99% (99 of 100)
F8a	99	Forward	98% (98 of 100)
R8a	99	Reverse	99% (99 of 100)
F8b	103	Forward	98% (98 of 100)
R8b	101	Reverse	98% (98 of 100)
F9b	106	Forward	>99% (103 of 104)
R9b	101	Reverse	100% (100 of 100)
F11b	107	Forward	>99% (103 of 104)
R11b	101	Reverse	100% (99 of 99)
F11c	107	Forward	>99% (103 of 104)
R11c	101	Reverse	100% (100 0f 100)

Table 24. Forward (F) and reverse (R) sequences from early results (study sample numbers 7-11) are compared to a reference database for Survivin PCR products. The reference sequence 'Ref Seq' result demonstrates the percentage of sequence homology between PCR product and the reference database.

Generally, there was excellent agreement between the PCR product and the reference database, with the majority of cases giving >98% sequence homology.

A green bar above a sequence position means a Phred quality score >20 (i.e., <1/100 chance of an error) whilst a red bar above a number indicates a higher (>1/100) chance of an error occurring when assigning a base – see below.

3.1.3.1.2 Finding the ROI

The sequence was assessed by ensuring an appropriate level of quality based on the Phred-called chance of an error – see below. Once sequences were deemed an acceptable level of quality, the region of interest was found using the search function on MacVector. For Survivin this was TCGC_GGA in the forward sequence and GTCC_GCG for the reverse direction with the underscore representing the -31 position of interest. Only when there was complementary agreement (i.e., C in the forward and G in reverse) would the status of the -31 position be ascertained.

3.1.3.1.3 Example Sequences

Chromatograms in figure 29 show a black waveform for G nucleotides (GG homozygote, top of image) a blue for C (CC homozygote, bottom image) and both a black and a blue waveform for the CG heterozygote (middle of fig 29). As can be seen in all three chromatograms, there is evidence of poor-quality data at the start of the sequence – as shown by the red bars (>1% chance of error) up to the ~10^{th} base in sequence. The addition of the 18 bases from the universal primer at the 5' sequence upstream of the F1R1 primer would have neatly removed this error-prone region. However, it was not technically feasible as the efficiency of the PCR reactions deteriorated when the extended primer was used.

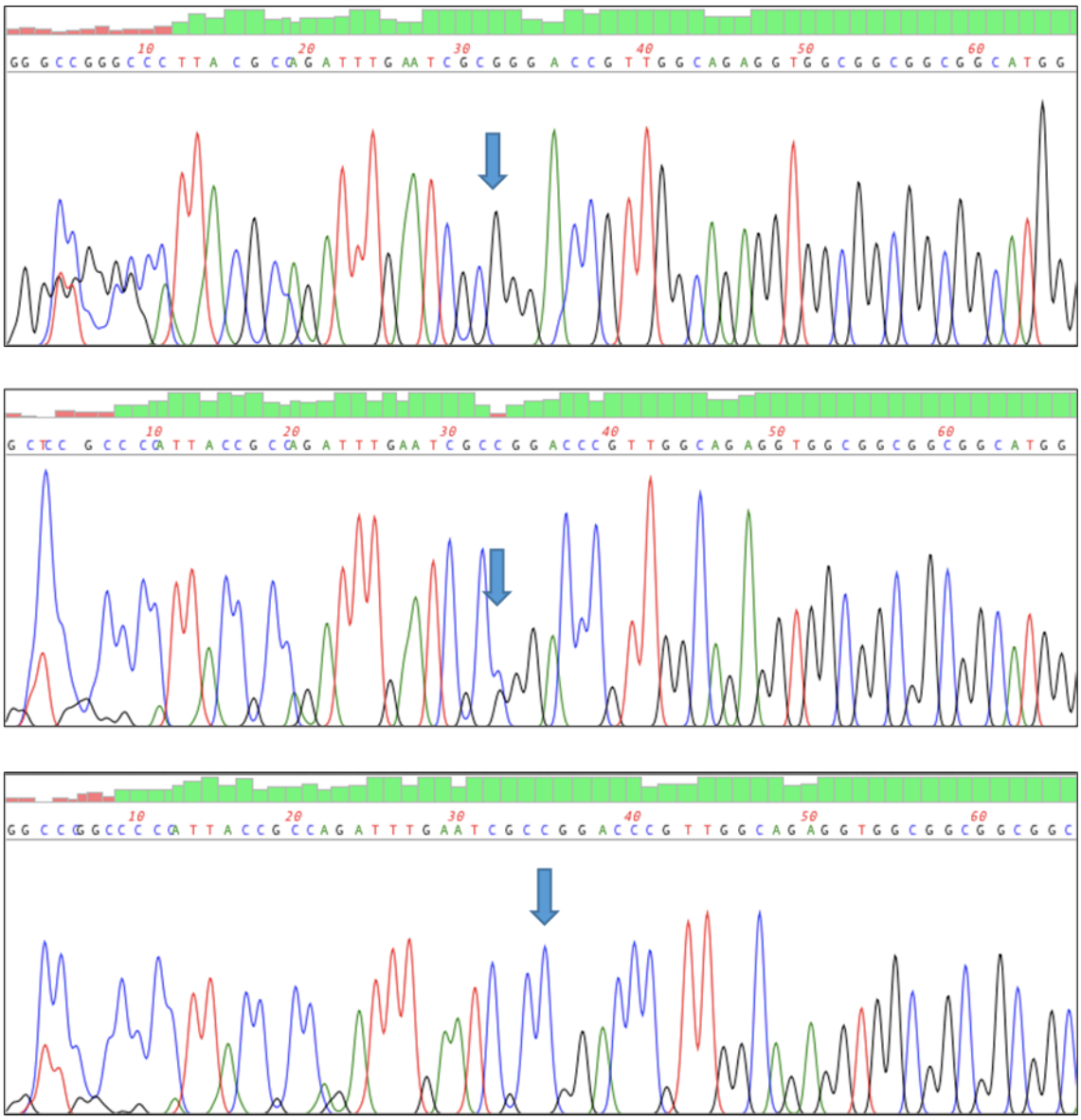


Figure 29. Chromatograms of forward sequences around the Survivin -31C/G position.

Chromatograms at the top (GG homozygote, position 32), middle (CG heterozygote, position 33) and bottom (CC homozygote, position 35) are seen in figure 29. The green bars over the sequence number indicates the 'phred' base-calling accuracy in measurement, with an assumed error rate of <1%. When a red bar is seen (as in the case in position 33 in the heterozygous middle sequence) the error rate is above this 1% threshold.

3.1.3.2 Telomerase Promoter

Telomerase sequencing reactions were processed after all the data regarding the Survivin -31G>C was known. This delay meant access to the MacVector software was re-purchased as the annual subscription had elapsed.

3.1.3.2.1 Quality Assurance

Regrettably an update to the software meant the BLAST sequence analysis tool did not function as previously described for the Survivin sequences. These original Survivin sequences were re-run and gave the same non-interpreted reading, as given for the new Telomerase sequences, as the software stalled. An example of this error can be seen in fig 30.

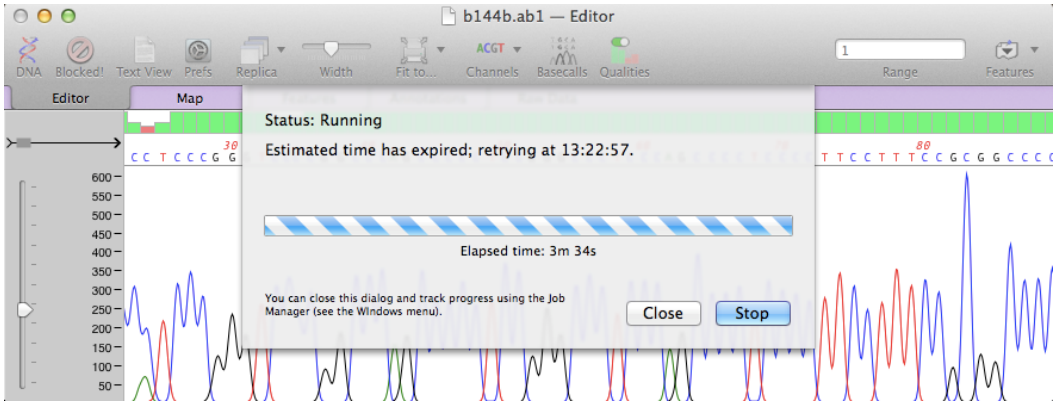


Figure 30. Analysis of the forward sequence from the Telomerase PCR products sample 144b using the '.ab1' format.

However, the Telomerase promoter sequences generated were generally a lot cleaner than those for Survivin, with significantly better base-calling ability when viewing the ".ab1" files. The 'phred' base call numerical values (denoted by the green box above the base) consistently gave quality scores >30 (i.e., 0.1% chance of error, 99.9% accurate) and regularly gave readings of 50 (0.001% error or 99.999% accurate) or more. An example of the quality assessment taken from a chromatogram report from the forward sequence of 144b is shown in table 25.

Chromatogram Raw Data report for 144b		
Index Position	**Base**	**Phred Quality**
26	C	52
27	C	45
28	C	40
29	G	40
30	G	52
31	G	52
32	T	52
33	C	32
34	C	32
35	C	32
36	C	45
37	G	45
38	G	45
39	C	52
40	C	40
41	C	45
42	A	40
43	G	45
44	C	52
45	C	55
46	C	55
47	C	52
48	C	52
49	T	45

Table 25 Sequences from the Telomerase promoter were of significantly higher quality, as measured by the 'phred quality' given by the MacVector software.

Shorter sequences of the PCR products were used as input queries on the BLAST search engine. The areas searched contained both of the positions of interest (-124 and -146) to ensure WT/mutational status was adequately checked (see below). An example of search results for ten samples is shown in table 26.

Sample	PCR Product Positions (size)	Sequence Orientation	Ref Seq Result	Duration of Search (minutes)
96a	20 -55 (36)	Forward	100% (36/36)	38
96a	70 – 110 (41)	Reverse	100% (41/41)	32
137b	20 -55 (36)	Forward	100% (36/36)	25
137b	70 – 110 (41)	Reverse	100% (41/41)	29
112b	20 -55 (36)	Forward	100% (36/36)	25
112b	70 – 110 (41)	Reverse	100% (41/41)	40
144b	20 -55 (36)	Forward	100% (36/36)	30
144b	70 – 110 (41)	Reverse	100% (41/41)	34
146a	20 -55 (36)	Forward	100% (36/36)	4
146a	70 – 110 (41)	Reverse	100% (41/41)	33

Table 26.Telomerase PCR products from samples are shown alongside their regions of interest, sequence orientation and the Ref Seq result (accuracy) are shown alongside the duration of the search of the NCBI database.

Assessing these shorter sequences still took some considerable time but confirmed primers had amplified the correct target DNA. Examples of these BLAST searches can be seen in figure 31.

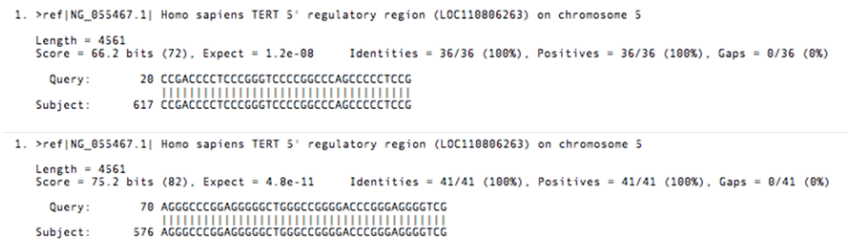


Figure 31. BLAST sequences for the Telomerase promoter are compared to NCBI reference

Figure 31 shows sample 112b with forward (upper image) and reverse (lower image) sequences confirming 100% homology.

3.1.3.2.2 Finding the ROI

The region of interest for the Telomerase promoter was different from that of Survivin as there were two targets in the same genetic sequence, as outlined in table 27.

Target	Forward Sequence	Reverse Sequence
-124	AGCCCC_TCCGGGCC	CCCGGA_GGGG
-146	ACCCCT_CCGGGT	ACCCGG_AGGGG

Table 27 Forward and reverse sequences for the mutational, or wild type, signatures in the Telomerase promoter region.

The mutational hotspots in the forward sequences tended to be roughly in the region of position 30 (-146) and 50 (-124) and for the reverse orientation around 80 (-124) and 100 (-146). The raw sequence data was searched using the targets outlined in table 27. The same approach of using the search function to find the regions of interest, whilst making a simultaneous quality assessment based on the Phred score, that has previously been adopted was used to ensure accuracy of base-calling of the neighbouring bases.

3.1.3.2.3 Example Sequences

Example sequences from the Telomerase promoter region PCR products can be seen in figure 32.

Figure 32. Chromatograms of Telomerase promoter regions, as visualised on MacVector.

A wild type promoter sequence is shown in the top sequence of figure 32, with blue C waveforms, with heterozygous CT alleles in the -124 (middle sequence, position 51) and the -146 position (bottom sequence, position 29).

3.2 Interpreting Promoter Status

Repeat experiments of either failed/un-interpretable runs were undertaken, maximising the data available for interpretation. Once as many sequences as possible were known, given the previously described limitations of using poor quality DNA, the un-blinding process was undertaken. On the rare occasions that repeated reactions yielded no

results, the unblinding process allowed these troublesome samples to be identified and a repeat cycle of randomisation, DNA retrieval, PCR reactions/analysis to be undertaken. The DNA was sometimes retrieved from the same block, or another tumour block to maximise the samples being studied. It was also relatively common that the background liver tissue block was either already, or was very nearly, exhausted due to the extensive IHC assessment of the liver tissues for the diagnostic work-up. When there was no background liver tissue available for research, only tumour samples were assessed.

3.2.1 Survivin Promoter

Excluding repeats, DNA from 261 samples underwent amplification with bi-directional confirmation of sequence in 254 samples (97.3%). This larger cohort includes the extrahepatic CCA. Of the 7 samples withdrawn from further analysis, one was withdrawn from the study as it is a spindle cell Cholangiocarcinoma and 6 others failed repeated attempts at PCR amplification.

When dCCA were excluded from analysis, sequences from 234 individual samples were attempted with 5 withdrawn from consideration (the spindle cell CCA and 4 that could not be amplified successfully) giving a similar sequencing success rate of 97.8% (229/234) across samples. The 4 failed samples were all from tumour tissues.

The Survivin promoter SNPs were fully concordant between background and tumour samples across all sequenced samples. This germline characteristic can be seen in figures 33-36. This is a surprising finding as it was expected that the -31 promoter SNP would mutate from WT in the background tissue. The pSurv CG heterozygotes (purple background), GG homozygotes (green background) and CC homozygotes (blue background) are highlighted in tumour (T) and background (BG) liver samples in figures 33 – 36.

Study Number	34a	40a	40b	45a	45c	46a	47a	47b	50a	50b	125a	125b
pSurv SNP	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG
Background/ Tumour	T	BG	T	BG	T	T	BG	T	BG	T	T	BG

Figure 33. Survivin -31G/C alleles in Mixed HCC-CCA and background liver.

Study Number	104a	104b	106a	106b	109a	109b	85a	85b	88a	88b	98a	98b	100a	100b	108a	108b	114a	114b	115a	115b
pSurv SNP	CG	CG	CG	CG	CG	CG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG
Background/ Tumour	T	BG	T	BG	BG	T	T	BG	BG	T	BG	T	BG	T	BG	T	T	BG	BG	T

Figure 34. Survivin -31G/C alleles in Adenoma and background liver.

Survivin Promoter: Hepatocellular Carcinoma

Study Number	7a	7b	8a	8c	10b	14a	14b	15a	15b	17a	17b	19a	19b	22a	22b	23a	23b	25a	25b	27a	27b	28a	28b	35a	35b	41a	41b	43a	43c	44a	44b	64a	64b	69a	70a	70b	73a	73b	74a	74b	116a	116x	131a	131b
pSurv SNP	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG
BG/ Tumour	BG	T	BG	T	T	T	T	BG	T	BG	T	BG	T	BG	T	BG	T	BG	T	BG	T	BG	T	T	BG	T	BG	BG	T	T	BG	T	BG	T	T	BG	BG	T	T	BG	BG	T	BG	T

Study Number	9b	9x	11a	11b	13a	13b	16a	16b	18a	18b	24a	24b	29a	29b	31a	31i	33a	33b	36a	36c	38a	38b	39a	39c	42x	42a	53b	54a	54b	55a	55b	56a	56b	57a	57b	71a	71b	72a	72b	93a	93b	101a	101b	117a	117b	119a	141a	141b	146a	146b
pSurv SNP	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG
BG/ Tumour	BG	T	BG	T	T	BG	T	BG	T	BG	BG	T	T	BG	BG	T	T	BG	T	BG	BG	T	T	T	T	BG	T	T	T	T	T	T	T	T	BG	BG	T	BG	T	BG	T	BG	T	T	BG	T	T	BG	T	BG

Study Number	20a	20b	26a	26b	30a	30b	37a	37b	118a	118b	143a	143b	147x
pSurv SNP	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC
BG/ Tumour	BG	T	BG	T	BG	T	BG	T	T	BG	T	BG	T

Survivin Promoter: Cholangiocellular Carcinoma

Study Number	49a	49b	51a	51b	60c	60d	62a	62b	65a	65b	76a	76b	78a	78b	79a	79b	80a	80b	84a	87a	87b	103a	103b	105a	107a	107b	120a	121a	124a	124b	135a	135b	137a	137b	138a	138b	142a	142b
pSurv SNP	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG
BG/Tumour	BG	T	BG	T	BG	T	BG	T	T	BG	T	BG	BG	T	T	BG	T	BG	T	T	BG	T	BG	T	BG	T	BG	T	BG	T	T	BG	BG	T	BG	T	T	BG

Study Number	48a	48b	52d	52e	58a	58b	68a	83a	99a	102a	102b	113a	113b	122a	126a	126b	129a	129b	144a	144b	77a	77b	90a	90b	112a	112b
pSurv SNP	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	CC	CC	CC	CC	CC	CC
BG/Tumour	BG	T	T	BG	BG	T	T	T	T	BG	T	BG	T	T	BG	T	BG	T	BG	T	BG	T	T	BG	BG	T

Figure 35. Survivin -31G/C alleles in Hepatocellular carcinoma and background liver.

Figure 36. Survivin -31G/C alleles in Cholangiocellular carcinoma and background liver.

The minor allele frequency for the -31G>C (*rs9904341*) Survivin promoter for C has been reported as between 0.31 – 0.47 (*Allele Frequency rs9904341 - SNP - NCBI*, 2020). From all samples sequenced in this cohort the minor allele frequency for C is 0.33, which is within these previously described limits.

3.2.2 Telomerase Promoter

For the Telomerase promoter, excluding repeats, DNA from a total of 271 individual tissues was retrieved. Five samples were withdrawn from this pool, comprising the spindle cell CCA and 4 samples with inconclusive results. Results were deemed inconclusive when there was a lack of agreement between bi-directional sequences. Therefor a total of 266 sequences (98.5%) of the original cohort of all liver neoplasms (i.e., including the extrahepatic CCA) were recorded. When the dCCA were excluded (leaving n=243), bi-directional sequences were taken from 98.8% (240/243) of samples processed. When more than one tumour sample was processed, a positive result was recorded if either tumour contained the -124/-146 mutation – with interesting inter and intra-tumoural heterogeneity that will be discussed below.

The telomerase promoter mutations were absent in all background tissues and only present in tumour samples of hepatocellular lineage. Intriguingly, there were no $pTERT^{Muts}$ in any Cholangiocarcinoma or Adenomas studied. All mutations were heterozygous, in agreement with the published literature (Cevik, Yildiz and Ozturk, 2015; Bell *et al.*, 2016). The $pTERT^{Muts}$ status of all neoplasms has been outlined in table 28.

Tumour	WT (%)	Mutant (%)
Hepatocellular Carcinoma	40 (69%)	18 (31%)
Cholangiocellular Carcinoma	36 (100%)	0 (0%)
Mixed HCC-CCA	6 (86%)	1 (14%)
Adenoma	10 (100%)	0 (0%)

Table 28. Telomerase promoter mutation rates across the primary hepatic neoplasms studied.

With 31.0% (18/58) of HCCs containing a pTERT mutant, this is similar to other reported mutation rates of 30-60% (H. W. Lee *et al.*, 2017; Nault *et al.*, 2019). For the purposes of simplification, HCC has included fibrolamellar HCC in the reported numbers in table 28 as one FL-HCC that contained a -124 promoter mutation (C228T). Should comparisons exclude FL-HCC, then similar rates (32.1%, 17/53) of pTERT are mutated in traditional HCCs. Of the 19 pTERT^{Muts} found, 17 (89.5%) were in the -124 position with 2 (10.5%) in the -146 position (C250T), in similar proportions to previously described (Nault *et al.*, 2013). Both mutations in this hotspot region were mutually exclusive, also in agreement with previous results (Vinagre *et al.*, 2013; Jiao *et al.*, 2018).

The pTERT^{Muts} in various neoplasms can be viewed in figures 37 - 40 with -124/ -146 mutants highlighted by a red/green box respectively in tumour (T) and background (BG) liver.

Mixed Tumours (Study Number)	34a	40a	40b	45a	45c	46a	47a	47b	50a	50b	125a	125b
pTERT WT/Mut										-124		
Background/ Tumour	T	BG	T	BG	T	T	BG	T	BG	T	T	BG

Figure 37 Telomerase promoter mutations in Mixed tumours.

Adenomas (Study Number)	104a	104b	106a	106b	109a	109b	85a	85b	88a	88b	98a	98b	100a	100b	108a	108b	114a	114b	115a	115b
pTERT WT/Mut																				
Background/ Tumour	T	BG	T	BG	BG	T	T	BG	BG	T	BG	T	BG	T	BG	T	T	BG	BG	T

Figure 38. Telomerase promoter mutations in Adenomas.

Telomerase Promoter: Hepatocellular Carcinoma

Study Number	pTERT WT/Mut	BG/Tumour
7a		BG T
7b	-124	BG T
8a		BG T
8c		BG T
9b		BG T
9x		BG T
10b		BG T
11a		BG T
11b		BG T
13a		BG T
13b		BG T
14a	-124	BG T
14b		BG T
15a		BG T
15b		BG T
16a		BG T
16b		BG T
17a		BG T
17b		BG T
18a		BG T
18b		BG T
19a		BG T
19b	-124	BG T
20a		BG T
20b		BG T
22a		BG T
22b	-124	BG T
23a		BG T
23b		BG T
24a		BG T
24b	-124	BG T
24x		BG T
25a		BG T
25b		BG T
26a		BG T
26b	-124	BG T
27a		BG T
27b		BG T
28a		BG T
28b		BG T
29a		BG T
29b		BG T
30a		BG T
30b		BG T
31a		BG T
31i		BG T
33a		BG T
33b		BG T
35a	-124	BG T
35b		BG T
36a		BG T
36c		BG T
37a		BG T
37b		BG T

Study Number	pTERT WT/Mut	BG/Tumour
38a		BG T
38b		BG T
39a		BG T
39c		BG T
41a		BG T
41b		BG T
42a		BG T
42x	-124	BG T
43a		BG T
43c		BG T
44a		BG T
44b		BG T
53b	-124	BG T
54a		BG T
54b		BG T
55a		BG T
55b		BG T
56a		BG T
56b		BG T
57a	-124	BG T
57b		BG T
64a		BG T
64b		BG T
69a		BG T
70a	-124	BG T
70b		BG T
71a		BG T
71b		BG T
72a		BG T
72b	-124	BG T
73a		BG T
73b	-146	BG T
74a		BG T
74b		BG T
93a		BG T
93b	-146	BG T
101a		BG T
101b	-124	BG T
116a		BG T
116x	-124	BG T
117a		BG T
117b		BG T
118a		BG T
118b		BG T
119a		BG T
131a		BG T
131b		BG T
141a	-124	BG T
141b		BG T
143a	-124	BG T
143b		BG T
146a		BG T
146b		BG T
147x		BG T

Telomerase Promoter: Cholangiocellular Carcinoma

Study Number	pTERT WT/Mut	BG/Tumour
48a		BG T
48b		BG T
49a		BG T
49b		BG T
51a		BG T
51b		BG T
52d		BG T
52e		BG T
58a		BG T
58b		BG T
60c		BG T
60d		BG T
62a		BG T
62b		BG T
65a		BG T
65b		BG T
68a		BG T
76a		BG T
76b		BG T
77a		BG T
77b		BG T
78a		BG T
78b		BG T
79a		BG T
79b		BG T
80a		BG T
80b		BG T
83a		BG T
84a		BG T
87a		BG T
87b		BG T
90a		BG T

Study Number	pTERT WT/Mut	BG/Tumour
90b		BG T
99a		BG T
102a		BG T
102b		BG T
103a		BG T
103b		BG T
105a		BG T
107a		BG T
107b		BG T
113a		BG T
113b		BG T
112a		BG T
112b		BG T
120a		BG T
121a		BG T
122a		BG T
124a		BG T
124b		BG T
126a		BG T
126b		BG T
129a		BG T
129b		BG T
135a		BG T
135b		BG T
137a		BG T
137b		BG T
138a		BG T
138b		BG T
142a		BG T
142b		BG T
144a		BG T
144b		BG T

Figure 39. Telomerase promoter mutations in Hepatocellular carcinomas.

Figure 40. Telomerase promoter mutations in cholangiocellular carcinomas.

It was relatively common for there to be more than one tumour in the HCC-liver resections. When more than one tumour was present within the resected tissues, or when there were two whole tumour blocks from a single patient, every effort was made to retrieve DNA and sequence the Telomerase promoter regions in these cases. Regrettably this was somewhat limited by some blocks being absent from the archive, but in 13 of the 52 HCCs it was possible to undertake this dual-sequencing. The results from available samples can be seen in figure 41.

Sample Number	7		8		13			14			19		24	30		39		42		116	140		143			145		
Tumour Number	T1	T2	T1	T2	T1	T2	T3	T1	T2	T3	T1	T2	T1	T1	T2	T1	T2	T1	T2	T1	T1	T2	T1	T2	T3	T1	T2	T3
WT/Mutant	-124									-124		-124	-124						-124	-124			-124		-124			

Figure 41. Intra-tumoural and inter-tumoural heterogeneity in tumour samples from 13 patients.

After initial difficulty sequencing tumour DNA from samples 24 & 116, tissue curls from a second tumour block were taken simultaneously when re-cutting the original tumour block. Somewhat surprisingly, intra-tumoural heterogeneity was found in these double-sequenced samples with both containing a WT and -124 pTERT mutation. An implication of this sampling bias being that the reported mutation rate may be higher than the 31% reported here, assuming satisfactory accuracy in the amplification and sequencing technique used.

Intriguingly, inter-tumoural heterogeneity was found in samples 7, 14, 19, 42 and 143 whilst samples 8, 13, 30, 39, 140 and 145 only contained $pTERT^{WT}$.

3.3 Statistical Analysis of Results

Statistical analysis was performed using IBM SPSS Statistics 24. Correlation between promoter status and clinical parameters were examined using Pearson's Chi squared test when there were >2 categorical dependent/independent variables. When both variables were nominal, Fishers exact test was used. The level of significance was set as 0.05 and

exact significance (two sided) been used throughout, with the Bonferroni method used to adjust p-values when comparing column proportions.

Overall survival was defined as the time from the operation and death, or last follow up set to 6th January 2020. Survival curves were plotted using Kaplan-Meier method and analysed with the Log-rank test.

Comparison between two groups was performed with the Mann-Whitney U-test as the outcome data was quantitative and non-normally distributed. Comparisons between three or more groups used the Kruskal-Wallis one-way analysis of variance (ANOVA) test for similar reasons. A P-value of <0.05 (two sided) was considered statistically significant.

3.3.1 Survivin Promoter

Pooled analysis of the results from the Survivin promoter sequencing across all neoplasia can be seen in table 29.

pSurv: All Neoplasms		N	CC	CG	GG	P-value
Gender	**Female**	44	3	22	19	0.954
	Male	65	5	34	26	
Outcome	**Alive**	51	4	25	22	0.914
	Dead	58	4	31	23	
Fibrosis	**Absent**	65	5	30	30	0.489
	Present	43	3	25	15	
Cirrhosis	**Absent**	86	6	39	41	**0.042**
	Present	22	2	16	4	
Viral Status	**No Infection**	94	6	47	41	0.342
	Viral Hepatitis	15	2	9	4	
Metabolic Risk Factors	**-**	56	5	29	22	0.835
	+	53	3	27	23	
Genetic Liver Disease	**-**	99	8	52	40	0.609
	+	10	0	5	5	
Tumour Type	**HCC**	50	4	23	23	0.109
	FL-HCC	6	1	2	3	
	CCA	36	3	21	12	
	Mixed HCC-CCA	7	0	7	0	
	Adenoma	10	0	3	7	

Table 29. Across all neoplasms studied, clinical measures are compared with alleles for the Survivin -31G/C.

When considering all neoplasms studied, the only statistically significant finding was that that fewer cirrhotic patients were GG homozygotes. Survivin alleles with GG homozygotes: 18.2% (4/22) have background liver cirrhosis, compared to 47.7% (41/86) of non-cirrhotically-derived liver tumours. In the CG heterozygotes, 72.7% (16/22) of the cirrhotics were CG heterozygotes compared with 45.3% (39/86) of the non-cirrhotics, χ^2 (2) = 6.341, p= 0.042. None of the other clinical measure differed significantly between Survivin promoter alleles.

3.3.1.1 All Malignancies

When excluding the benign adenomas, comparisons between clinical and pathological characteristics and the pSurv alleles can been seen in table 30. There were no significant results. Viral hepatitis, metabolic risk factors and genetic liver disease also failed to demonstrate any significant differences and have not been shown in table 30.

pSurv: Malignancies		N	CC	CG	GG	P-value
Gender	**Female**	35	3	20	12	0.900
	Male	64	5	33	26	
Outcome	**Alive**	41	4	22	15	0.863
	Dead	58	4	31	23	
Margin	**R0**	57	5	29	23	0.741
	R1	41	3	24	14	
Fibrosis	**Absent**	58	5	28	25	0.519
	Present	40	3	24	13	
Cirrhosis	**Absent**	76	6	36	34	0.078
	Present	22	2	16	4	
Tumour Stage	**I & II**	65	4	38	23	0.258
	III & IV	25	4	11	10	
Grade	**Well Diff**	11	0	7	4	0.396
	Mild/Mod Diff	65	8	33	24	
	Poorly Diff	17	0	11	6	
Vascular Invasion	**No VI**	42	3	24	15	0.861
	VI	51	5	27	19	
	+	10	0	5	5	
Tumour Type	**HCC**	50	4	23	23	0.168
	FL-HCC	6	1	2	3	
	CCA	36	3	21	12	
	Mixed HCC-CCA	7	0	7	0	

Table 30. Across all malignancies studied, clinical measures are compared with alleles for the Survivin -31G/C.

A non-significant difference in the distribution of GG homozygotes in the non-cirrhotic/cirrhotic groups is demonstrated in table 30. Nearly 20% of tumours (4/22,

18.2%) that develop in the context of cirrhosis are GG homozygotes, compared to 44.7% (34/76) of malignancies from non-cirrhotic livers, χ^2 (2) = 5.200, p= 0.078. When comparing Survivin promoter variants across tumour type, 46.0% (23/50) of HCCs, 33.3% (12/26) of CCAs and 0.0% (0/7) of Mixed tumours contain GG homozygotes, $\chi^2(6) = 8.974$, p= 0.168.

3.3.2 Survivin Promoter & Tumour Subtype

3.3.2.1 HCC+FL-HCC

There were no associations found between clinical measures and -31G/C Survivin promoter genotypes in either the grouped HCC + FL-HCC, as outlined in table 31, or HCC subgroups (data not shown). The analysis of pSurv and corresponding subgrouping of alleles does not yield any significant correlations with clinical disease in all HCCs.

pSurv: HCC & FL-HCC		N	CC	CG	GG	P-value
Gender	**Female**	13	2	6	5	0.651
	Male	43	3	19	21	
Outcome	**Alive**	28	3	13	12	0.859
	Dead	28	2	12	14	
Margin	**R0**	39	4	16	19	0.619
	R1	16	1	9	6	
Fibrosis	**Absent**	31	3	13	15	0.923
	Present	25	2	12	11	
Cirrhosis	**Absent**	41	3	16	22	0.212
	Present	15	2	9	4	
Tumour Stage	**I & II**	31	1	15	15	0.093
	III & IV	18	4	8	6	
Grade	**Well Diff**	10	0	6	4	0.645
	Mild/Mod Diff	36	5	16	15	
	Poorly Diff	6	0	3	3	
Vascular Invasion	**No VI**	31	2	15	14	0.772
	VI	21	3	10	8	

Table 31. Across all HCC subtypes studied, clinical measures are compared with alleles for the Survivin -31G/C.

Most HCCs were stage I/II and CC homozygotes comprised 3.2% of these tumours, however of the 18 stage III/IV HCCs the CC allele accounted for 22.2% of these, χ^2 (2) = 4.667, p= 0.093. Otherwise, no significant difference in the distribution of alleles was found when comparing clinical parameters.

3.3.2.2 Cholangiocarcinoma & pSurv

As there were no $pTERT^{Mut}$ in Cholangiocarcinoma, the analysis of promoter sequences was limited to pSurv. I found no significant correlations when comparing pSurv and clinically recorded data, as outlined in tables 32. Subgroup analysis, including the effect of dominant alleles (data not shown) also failed to find any significant difference between clinical parameters.

Unique to CCA tumours is the presence (or otherwise) of perineural invasion as well as the location (iCCA vs. pCCA) that has been routinely reported upon in the clinical records. However, there was no significant distribution in the alleles of the Survivin promoter SNP when considering any clinical parameter. When the iCCAs were assessed, there was also no difference in the distribution of the pSurv alleles, data not shown.

pSurv: CCAs		N	CC	CG	GG	P-value
Gender	**Female**	20	1	12	7	0.774
	Male	16	2	9	5	
Outcome	**Alive**	12	1	8	3	0.862
	Dead	24	2	13	9	
Margin	**R0**	12	1	7	4	1.000
	R1	24	2	14	8	
Fibrosis	**Absent**	26	2	14	10	0.885
	Present	9	1	6	2	
Cirrhosis	**Absent**	34	3	19	12	1.000
	Present	1	0	1	0	
Tumour Stage	**I & II**	27	3	16	8	0.359
	III & IV	7	0	3	4	
Grade	**Well Diff**	1	0	1	0	0.685
	Mild/Mod Diff	24	3	12	9	
	Poorly Diff	9	0	6	3	
Vascular Invasion	**No VI**	8	1	6	1	0.308
	VI	26	2	13	11	
Perineural Invasion	**-**	18	2	12	4	0.446
	+	18	1	9	8	

Table 32. Across all CCAs studied, clinical measures are compared with alleles for the Survivin -31G/C.

3.3.2.3 Mixed

As all seven of the mixed tumours were found to be CG heterozygotes, statistical analysis of differences in clinical/pathological findings was unable to be undertaken.

3.3.2.4 Adenoma

The benign adenomas clearly did not possess malignant behaviours (vascular invasion, stage, grade, etc) and so these inappropriate measures have been removed from the comparisons outlined in tables 33. There were also no CC homozygotes in the studied tumours, meaning the results are essentially the same and that comparing CC to CG+GG is not possible.

pSurv: Adenoma		N	CC	CG	GG	P value
Gender	Female	9	0	2	7	0.300
	Male	1	0	1	0	
Fibrosis	Absent	7	0	2	5	1.000
	Present	3	0	1	2	
Metabolic Risk Factors	+	8	0	2	6	1.000
	-	2	0	1	1	

Table 33. Clinical measures are compared in the Adenomas, depending on the Survivin -31G/C alleles.

3.3.3 Discussion

With the surprising result of 100% concordance between tumour and background liver tissues for Survivin's rs9904341 across all samples there was a distinct lack of clinically meaningful correlations subsequent to this. The status of the Survivin -31 SNP should be considered a germline characteristic, rather than a somatically altered mutation that facilitates tumourigenesis.

When the pooled results were interpreted the single statistically significant result is the Survivin GG homozygote occurring more often in non-cirrhotic livers that facilitated the growth of both benign and malignant neoplasms (p=0.042). When considering all malignant tumours, and their pSurv status, the distribution of GG tumours did not differ between cirrhotic and non-cirrhotic liver tissues, χ^2 (2) = 5.200, p= 0.078.

The significance these results is of limited value in this small cohort of patients. The GG allele did not correlate with any tumour type across all neoplasms (p= 0.109) or in the malignant cohort, p= 0.168. Given that GG homozygous patients have previously been shown to be associated with an increased risk of solid organ malignancy (in

NSCLC at least) my findings should not be overstated, rather interpreted with caution (Aynaci *et al.*, 2013).

Otherwise, the Survivin promoter SNPs did not correlate with any other clinical or pathological characteristic that was measured. Analysing Survivin promoter status in primary liver cancer is not a useful tool, based on the evidence seen here.

3.3.4 Telomerase Promoter

Given the more straightforward results from the pTERT sequences (heterozygous mutation or WT only) statistical analyses were more limited when compared to pSurv as can be seen from table 34. For the purposes of data analysis both the -146 and -124 mutations were reported together as a 'mutant', in line with previous publications (Horn *et al.*, 2013; Huang *et al.*, 2013).

pTERT: All Neoplasms		**N**	**Mutant**	**WT**	**P value**
Gender	**Female**	45	1	44	**0.001**
	Male	66	18	48	
Outcome	**Alive**	51	6	45	0.210
	Dead	60	13	47	
Fibrosis	**-**	66	6	60	**0.009**
	+	44	13	31	
Cirrhosis	**-**	87	10	77	**0.004**
	+	23	9	14	
Viral Hepatitis	**-**	96	14	82	0.131
	+	15	5	10	
Metabolic Risk Factors	**-**	57	8	49	0.453
	+	54	11	43	
Genetic Liver Disease	**-**	101	17	84	0.680
	+	10	2	8	
Tumour Type	**HCC**	35	17	52	**0.002**
	FL-HCC	6	1	5	
	CCA	36	0	36	
	Mixed HCC-CCA	7	1	6	
	Adenoma	10	0	10	

Table 34. Clinical measures are compared between mutants and wild type Telomerase promoter across all neoplasms.

From table 34 there are a few interesting correlations to report when considering Telomerase promoter mutations across the whole study cohort. Of the 19 $pTERT^{Muts}$ present, 18 occurred in men. Mutations occurred more often in 27.3% of men's cancers

compared to 2.2% of female patients, p= 0.001. There were more pTERT^{Muts} that grew on a background of fibrosis (29.5%) compared to non-fibrosis-derived liver tumours (9.1%, p= 0.009). Perhaps indicating a correlation with early tumour development. Telomerase promoter mutations also occurred more frequently in the context of background liver cirrhosis (39.1%) compared to non-cirrhotically derived liver tumours (11.5%, p= 0.004). Finally, pTERT^{Muts} appear to be an HCC-lineage specific mutation, with 17/19 occurring in HCCs (χ^2 (4) = 18.432, p= 0.002), one case occurring in FL-HCC and the final pTERT^{Muts} being in a mixed HCC-CCA tumour. There were no pTERT^{Muts} in either the CCAs or the Adenomas in this cohort. There was no difference in the distribution of the clinical parameters: viral hepatitis (p= 0.131), metabolic disease (p= 0.453) and genetic liver disease (p= 0.680) so these parameters will not be discussed further.

When excluding benign adenomas from consideration, pTERT^{Muts} occur more often in men (27.7%) compared to women (2.8%, p= 0.001). Across all malignancies pTERT^{Muts} occurs in 31.7% of patients with liver fibrosis compared with 10.2% in non-fibrotic hepatic tissue, p= 0.01. Non-cirrhotic livers yield tumours with pTERT^{Muts} in 13.0% of cases compared to 39.1% of cirrhotic livers (p= 0.012).

Across all malignancies, pTERT^{Muts} occur more frequently in HCCs compared with CCA and Mixed tumours (χ^2 (3) = 15.013, p= 0.003). However, in the malignant cohort there was no significant correlation with all-cause mortality, positive surgical margin, tumour stage/grade or vascular invasion. Risk factors such as viral hepatitis, metabolic risk factors or genetic liver disease also failed to demonstrate any correlation with pTERT^{Muts}. When excluding the benign neoplasms, table 35 highlights the results from the pooled analyses.

pTERT: All Malignancies		N	Mutant	WT	P value
Gender	**Female**	36	1	35	**0.001**
	Male	65	18	47	
Outcome	**Alive**	41	6	35	0.444
	Dead	60	13	47	
Margin	**R0**	58	14	44	0.196
	R1	41	5	36	
Fibrosis	**-**	59	6	53	**0.01**
	+	41	13	28	
Cirrhosis	**-**	77	10	67	**0.012**
	+	23	9	14	
Tumour Stage	**I & II**	65	9	56	0.221
	III & IV	26	7	19	
Grade	**Well Diff**	11	2	9	0.401
	Mild/Mod Diff	66	14	52	
	Poorly Diff	17	1	16	
Vascular Invasion	**-**	42	7	35	0.794
	+	52	10	42	
Tumour Type	**HCC**	35	17	52	**0.003**
	FL-HCC	6	1	5	
	CCA	36	0	36	
	Mixed HCC-CCA	7	1	6	

Table 35. Clinical measures are compared between mutants and wild type Telomerase promoter sequences across all malignancies.

3.3.5 Tumour Specific Analyses

With pTERT found almost exclusively in HCCs, subgroup analysis of all HCCs (i.e., including FL-HCC, table 36) and traditional HCCs (excluding FL-HCCs, table 37) was undertaken.

pTERT: HCC & FL-HCC		**N**	**Mutant**	**WT**	**P value**
Gender	**Female**	14	0	14	**0.003**
	Male	44	18	26	
Outcome	**Alive**	28	5	23	**0.049**
	Dead	30	13	17	
Margin	**R0**	40	13	27	1.000
	R1	16	5	11	
Fibrosis	**-**	32	6	26	**0.044**
	+	26	12	14	
Cirrhosis	**-**	42	10	23	0.066
	+	16	8	8	
Tumour Stage	**I & II**	31	8	23	0.528
	III & IV	19	7	12	
Grade	**Well Diff**	10	2	8	0.519
	Mild/Mod Diff	37	13	24	
	Poorly Diff	6	1	5	
Vascular Invasion	**-**	31	6	25	0.068
	+	22	10	12	

Table 36. Clinical measures are compared between mutants and wild type Telomerase promoter sequences across all HCCs.

Table 36 reports pTERT^{Muts} in HCC & FL-HCC showing a strong male predominance with 40.9% of men harbouring a mutation compared with 0.0% of women, p= 0.003. There is also a weaker, although still significant, association with fibrosis (46.2% of tumours that arise in the context of fibrosis yield pTERT^{Muts} compared with 18.8% of

non-fibrotically derived tumours, p= 0.044. It's interesting that the highest rate of finding $pTERT^{Muts}$ in HCCs is in those with fibrotic liver disease as it maybe the aetiology of HCC is different in those with a chronic liver disease with a longer duration giving more time for the mutation to emerge and predispose to HCC. The inter-relationship between cirrhosis and $pTERT^{Muts}$ is less certain as 50.0% of cirrhotics have a mutation, compared with 23.8% of non-cirrhotic patients, p= 0.066.

There is also a new association with all-cause mortality as 43.3% of those who die have a $pTERT^{Muts}$ compared with 17.9% of those who live, p= 0.049. However, this finding is of marginal significance given the smaller study numbers and the relatively large p-value.

All other clinical and histopathological characteristics did not demonstrate a significant difference between the $pTERT^{Mut/WT}$ groups. Vascular invasion occurs more often (although non-significantly so) in 45.5% of tumours with $pTERT^{Muts}$, compared with 19.4% of non-mutated tumours, p= 0.068.

When FL-HCC are excluded and traditional HCC's $pTERT^{Muts}$ status is assessed, the results are shown in table 37. Mutations occur in 42.7% of male patients, compared with 0.0% of females, p= 0.005. Of those who have died, 44.8% harbour a $pTERT^{Muts}$ containing tumour, compared with 17.4% of patients still alive, p= 0.043. Tumours that invade the vasculature have a higher $pTERT^{Muts}$ rate (52.6%) compared with non-invasive tumours (20.17%, p= 0.031).

In classical HCCs $pTERT^{Muts}$ occur in the context of fibrosis in 46.2% of patients, compared with 19.2% of those with non-fibrotic liver tissue, p= 0.075. And cirrhotics have a $pTERT^{Muts}$ rate of 50.0%, compared with 25.0% in non-cirrhotics, p= 0.111. Both of these previous measures were significantly associated with $pTERT^{Muts}$, but in

the smaller sample size these are no longer significantly correlated, albeit with a convincing trend for pTERT^{Muts} occurring in the context of fibrosis.

pTERT: HCC only		N	Mutant	WT	P value
Gender	**Female**	12	0	12	**0.005**
	Male	40	17	23	
Outcome	**Alive**	23	4	19	**0.043**
	Dead	29	13	16	
Margin	**R0**	36	12	24	1.000
	R1	14	5	9	
Fibrosis	-	26	5	21	0.075
	+	26	12	14	
Cirrhosis	-	36	9	27	0.111
	+	16	8	8	
Tumour Stage	**I & II**	29	8	21	0.516
	III & IV	17	7	10	
Grade	**Well Diff**	8	2	6	0.689
	Mild/Mod Diff	35	13	22	
	Poorly Diff	5	1	4	
Vascular Invasion	-	29	6	23	**0.031**
	+	19	10	9	

Table 37. Across traditional HCCs, clinical measures are compared between mutants and wild type Telomerase promoter sequences.

3.3.5.1 Mixed

In the small cohort of seven mixed tumours there were no significant relationships between clinical or pathological data and the pTERT^{Mut}. This is perhaps due, at least in part, to the small number of mixed tumours studied.

3.3.6 Discussion

My results demonstrate that pTERT^{Muts} are an HCC-lineage specific somatic mutation (p= 0.001), are associated with male gender (p=0.001), and adverse clinical measures when compared across the entire study cohort. When comparing malignant liver tumours pTERT^{Muts} remain a male phenomenon (p= 0.001), occur more frequently in HCCs (p= 0.002), occur more frequently in the context of fibrotic (p= 0.01) and cirrhotic (p= 0.012) background liver tissues. Previously published data has also reported an absence of pTERT^{Muts} in CCA, in agreement with our findings (Quaas *et al.*, 2014).

The role of chronic inflammation selecting for pTERT^{Muts} in malignancies is only significant when considering the whole tumour cohort and not in tumour sub-type analyses. This is possibly a reflection of the selection bias in patients offered surgery (fewer than expected patients with cirrhosis) or the small cohort of patients being studied. This aetiology of PLCs must also be considered with the established links between fibrosis, cirrhosis and HCC development compared to the much more sporadic neoplastic nature of CCAs. These differences could potentially explain how some correlations are apparent when considering larger groups (all hepatic neoplasms/malignancies and all HCC subtypes) but are absent when only considering classical HCCs. As fibrolamellar HCCs are histologically and genetically very different to traditional HCCs comparisons have been made assessing if there are differences when FL-HCCs are included/excluded. However, when comparing all HCC subtypes (including FL-HCC) there is a significant relationship between pTERT^{Mut} and fibrosis (p=0.044), but no significant relationship with tumourigenesis in the context of cirrhotic liver tissues, p= 0.066. Furthermore, when assessing all HCCs the male association endured (p= 0.003) as did the correlation with all-cause mortality, p= 0.049.

When FL-HCC tumours are excluded, an HCC tumour specific analyses confirms the strong male association with $pTERT^{Muts}$ (p=0.005) and demonstrates weak, albeit statistically significant, correlations with vascular invasion (p= 0.031) and all-cause mortality (p= 0.043).

Male preponderance of $pTERT^{Muts}$ has been previously reported in HCCs but no significant correlation for vascular invasion was reported in their cohort (Nault *et al.*, 2013). Work from a Korean cohort larger than ours (but still using FFPE tissues as a DNA source) have found $pTERT^{Muts}$ preferentially occurring in men (p= 0.027) and in 28.8% of HCCs (H. W. Lee *et al.*, 2017).

When considering non-hepatic tumours, $pTERT^{Muts}$ have been associated with adverse clinical measures including vascular invasion in a recent meta-analysis of 11,382 cases of thyroid carcinoma (Yang *et al.*, 2020). My work has increased the number of HCCs under study and also shown a similar association, indicating altered tumour biology based on a promoter mutation.

3.3.7 Comparisons between Groups

The continuous variables of age (years) and tumour size (mm) were recorded with comparisons made between promoter status overall, and at the level of tumour subtype.

3.3.7.1 All Neoplasms

No significant relationships were found between pSurv/ pTERT status and age or tumour size across all neoplasms, as outlined in table 38.

All Neoplasms		N	Tumour Size (mm)				Age (years)		
			Mean Rank	Mean +/- S.D.	Median		Mean Rank	Mean +/- S.D.	Median
pSurv	**CC**	8	51.0	59.5 +/- 19.2	67.5		54.4	66.6 +/- 37.6	55.0
	CG	56	56.0	62.6 +/- 12.9	64.0		56.6	78.3 +/- 53.1	70.0
	GG	45	54.4	59.8 +/- 15.8	63.0		53.1	72.5 +/- 46.1	60.0
	χ^2(2)		0.205				0.296		
	p-value		0.903				0.862		
pTERT	**WT**	92	58.4	77.8 +/- 48.0	63.0		53.4	60.0 +/- 14.8	63.0
	Mut	19	44.4	64.0 +/- 59.3	45.0		68.4	66.8 +/- 11.7	69.0
	U		653.0				1100.0		
	p-value		0.083				0.065		

Table 38. For all neoplasms, clinicopathological characteristics are analysed based on the promoter mutational status of pSurv and pTERT.

When comparing all neoplasms, a Kruskal-Wallis test showed that there was no significant difference in tumour size based on the Survivin promoter variants, $\chi^2(2) = 0.205$, p= 0.903, with mean rank scores of 51.0 for the CC homozygote, 56.0 for the CG heterozygote and 54.4 for the GG homozygote. Similarly, there was no significant difference in patient age based on the Survivin promoter variants, χ^2 (2) = 0.296, p= 0.862, with a mean rank of 54.4 (CC), 56.6 (CG) and 53.1 (GG).

A Mann-Whitney U test demonstrated that $pTERT^{Muts}$ tended to occur in smaller tumours (median size 45mm, mean rank 44.37) compared to wild type (median size 63mm, mean rank 58.4) but this was not a significant difference, U= 653.0, p= 0.083. With a median age of 69 years (mean rank 68.4) $pTERT^{Muts}$ tended to develop in develop in older patients, compared to $pTERT^{WT}$, whose median age was 63 (mean rank 53.4) however this was not a statistically significant difference, U= 1100.0, p= 0.065. This data will have been skewed by the presence of the benign adenomas, which are assessed below.

3.3.7.2 All Malignancies

Grouped into malignant neoplasms, results are shown in table 39.

All Malignancies		N	Tumour Size (mm)				Age (years)		
			Mean Rank	Mean +/- S.D.	Median		Mean Rank	Mean +/- S.D.	Median
pSurv	**CC**	8	47.3	66.6 +/- 37.6	55.0		46.2	59.5 +/- 19.2	67.5
	CG	53	51.9	79.5 +/- 53.9	70.0		49.4	64.0 +/- 11.5	65.0
	GG	38	48.0	69.8 +/- 46.5	60.0		51.6	63.9 +/- 13.0	67.5
	$\chi^2(2)$		0.482				0.281		
	p-value		0.786				0.869		
pTERT	**WT**	92	53.4	77.8 +/- 48.9	65.0		49.2	62.8 +/- 12.8	66.0
	Mut	19	40.7	64.0 +/- 59.3	45.0		58.7	66.8 +/- 11.7	69.0
	U		583.5				925.0		
	p-value		0.089				0.204		

Table 39. For all malignant neoplasms, clinicopathological characteristics are studied based on the promoter mutational status of pSurv and pTERT.

When comparing all malignant neoplasms, a Kruskal-Wallis test showed that there was no significant difference in tumour size based on the Survivin promoter variants, $\chi^2(2) = 0.482$, p= 0.786, with mean rank scores of 47.3 for the CC homozygote, 51.9 for the CG heterozygote and 48.0 for the GG homozygote. Similarly, there was no significant difference in patient age based on the Survivin promoter variants with a mean rank scores of 46.2 (CC), 49.4 (CG) and 51.6 (GG), χ^2 (2) = 0.281, p= 0.869.

Interestingly, $pTERT^{Muts}$ occurred in smaller malignant liver tumours (median size 45mm, mean rank 40.7) when compared with $pTERT^{WT}$ tumours (median 65mm, mean rank 53.4) but this difference was not statistically significant, U= 583.5, p= 0.089. This finding is perhaps more representative of the role Telomerase plays in smaller malignant tumours, when considering the entire malignant cohort, but any further associations in tumour sub-group analysis may lack the study numbers required to fully describe this.

The median age for patients who develop a Telomerase mutation was 69 (mean rank 58.7) which is older than patients with WT tumours (median 66, mean rank 49.2) but this difference was not significant, U= 925.0, p= 0.204.

3.3.7.2.1 HCC& FL

When comparing all HCCs, a Kruskal-Wallis test showed that there was no significant difference in tumour size based on the Survivin promoter variants, $\chi^2(2) = 0.796$, p= 0.672, with mean rank scores of 29.7 for the CC homozygote, 30.4 for the CG heterozygote and 26.4 for the GG homozygote. Similarly, there was no significant difference in patient age based on the Survivin promoter variants, $\chi^2(2) = 1.907$, p= 0.385, with a mean rank scores of 18.9 (CC), 29.6 (CG) and 29.3 (GG).

A Mann-Whitney U test showed that pTERT status had no significant effect on tumour size with WT tumours tending to be larger (median size 82.5mm, mean rank 31.7) compared to the mutants (median size 45.5, mean rank 24.6), but this was not a statistically significant difference, U= 271.5, p= 0.137. Wild type tumours occurred in younger patients (median age 66, mean rank 28.0) when compared to mutant tumours (median age 70, mean rank 32.8), but this difference also failed to reach significance, U= 419.0, p= 0.321.

3.3.7.2.2 HCC Only

When comparing classical HCCs, a Kruskal-Wallis test showed that there was no significant difference in tumour size based on the Survivin promoter variants, $\chi^2(2) = 1.117$, p= 0.572, with mean rank scores of 30.3 for the CC homozygote, 26.8 for the CG heterozygote and 23.6 for the GG homozygote. Similarly, there was no significant difference in patient age based on the Survivin promoter variants, $\chi^2 (2) = 1.306$, p= 0.520, with mean ranks of 17.6 (CC), 25.8 (CG) and 26.6 (GG).

A Mann-Whitney U test demonstrated that pTERT^{Muts} status had no significant effect on tumour size. Wild type HCCs were larger (median 80mm, mean rank 28.5) compared to mutants (median 45mm, mean rank 22.4) but not significantly so, U= 228.0, p= 0.175. Telomerase mutations occurred in HCCs from older patients (median age 72, mean rank 28.5) when compared with their WT counterparts (median age 67, mean rank 25.5) but lacked statistical significance, U= 331.0, p= 0.513.

3.3.7.2.3 CCA

When comparing all CCAs, a Kruskal-Wallis test showed that there was no significant difference in tumour size based on the Survivin promoter variants, $\chi^2(2) = 1.109$, p= 0.574, with mean rank scores of 12.7 for the CC homozygote, 19.5 for the CG heterozygote and 18.3 for the GG homozygote. Similarly, there was no significant difference in patient age based on the Survivin promoter variants, $\chi^2 (2) = 1.600$, p= 0.449, with mean ranks of 25.8 (CC), 17.7 (CG) and 18.1 (GG).

3.3.7.2.4 Adenoma

A Mann-Whitney U test showed that the two pSurv variants present in the 10 benign tumours had no significant correlation with Adenoma size (U= 13.0, p= 0.667), and was not correlated with patient age (U= 9.0, p= 0.833).

3.3.8 Discussion

There is no significant difference between either Survivin promoter variants, or Telomerase promoter mutants, when comparing tumour size or patient age. However, when considering the whole cohort, median pTERT^{Muts} were smaller compared to WT (p= 0.083) and occurred in older patients, p= 0.065.

My findings from the HCC tumour sub-group analyses (both including and excluding FL-HCCs) found that pTERT^{Muts} occurred (non-significantly) in older patients, and in smaller tumours, similar to others findings. Previously published work regarding

pTERT^{Mutant} tumours found them to be smaller (<5cm, p=0.01) and in older patients (>60 years old, p=0.08) (Nault *et al.*, 2013). However, Nault's work only assessed HCCs (as opposed to all hepatic neoplasms, including FL-HCC) and the significance of these differences was not reflected in the tumour subtype analysis.

3.3.9 Survival Analysis

Survival analysis was undertaken by plotting Kaplan-Meier curves using the Log Rank (Mantel-Cox) method for overall survival. When quoted the average survival data is recorded in the following manner: mean survival +/- standard error (95% confidence interval). There was extremely limited data in the clinical records of patient specific data, such as disease-free survival (dfs) or progression free survival (pfs) so only overall survival has been recorded.

3.3.9.1 All Neoplasms

Kaplan-Meier survival analyses for all neoplasm and their respective pTERT & pSurv status can been seen in figure 42. There were no significant associations across the whole cohort.

There was no difference in survival between pTERT$^{Mut/WT}$, as shown in the Kaplan-Meier plot (top left) in figure 42, χ^2 (1) = 0.819, p= 0.366. Average survival data for WT tumours include a mean survival time of 81.5 +/- 7.4 months (95% CI 67.1 – 96.0) and median survival time of 76.0 +/- 27.0 months (95% CI 23.0 – 129.0). Patient with pTERT^{Muts} had a mean survival time of 65.7 +/- 14.2 months (95% CI 37.9 – 93.5) and a corresponding median survival of 42.0 +/- 12.9 months (95% CI 16.7 – 67.3). Cox regression analysis also failed to demonstrate any correlation between Survival and pTERT$^{Mut/WT}$ status.

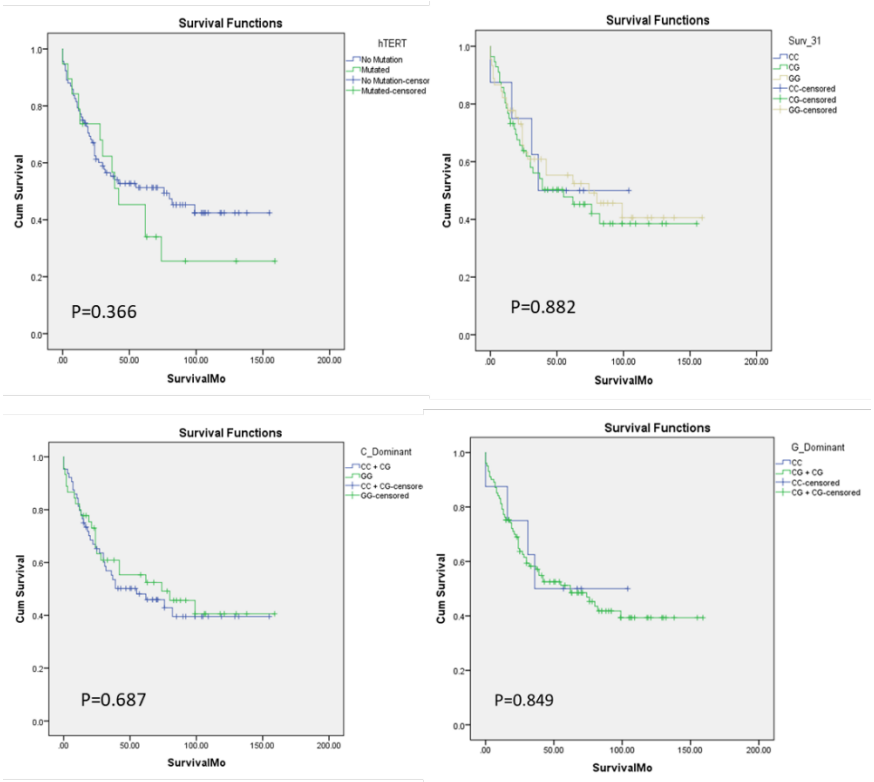


Figure 42. Cumulative survival based on Telomerase/ Survivin promoter status across all

There was also no difference in cumulative survival based on the Survivin promoter allele status, χ^2 (2) = 0.251, p= 0.882. Average survival data for CC homozygotes includes a mean survival duration of 62.4 +/- 15.1 months (95% CI 32.7 – 92.0) with no median survival data fewer than 50% of these patients died. CG heterozygotes had a mean survival of 76.6 +/- 9.2 months (95% CI 58.5 – 94.6) and median survival of 55.0 +/- 21.7 months (95% CI 12.5 – 97.4). GG homozygotes had a mean survival of 84.0 +/- 10.7 months (95% CI 63.1 – 104.9) and median survival of 74.0 +/- 30.9 months (95% CI 13.4 – 134.6.

When grouping C-containing alleles together (so-called *C-dominance*) there was no difference in Survival, χ^2 (1) = 0.163, p= 0.687. The GG homozygotes had a mean survival of 84.0 +/- 10.7 months (95% CI 63.1 – 104.9) and median survival of 74.0 +/- 30.1 (95% CI 13.4 – 134.6) compared with the grouped (CC+CG) mean survival of 77.7+/- 8.7 months (95% CI 60.7 – 94.7) and median survival of 55.0 +/- 20.9 months (95% CI 14.1 – 95.9).

Comparing the CC homozygotes with *G-dominant* alleles found no significant difference on survival, χ^2 (1) = 0.036, p= 0.849. Mean survival for CC homozygotes was 62.4 +/- 15.1 months (95% CI 32.7 – 92.0). The G-dominant alleles had a mean survival of 80.7 +/- 7.1 months (95% CI 66.8 – 94.6) and median survival of 62.0 +/- 16.6 months (95% CI 29.6 – 94.4). Subsequent analyses in tumour subtypes also failed to find any correlation between allele-dominance and overall survival and have not been show in the following figures.

3.3.9.2 All Malignancies

When excluding the benign adenomas, the malignant neoplasms were considered together, as shown in figure 43, with no significant difference in cumulative survival.

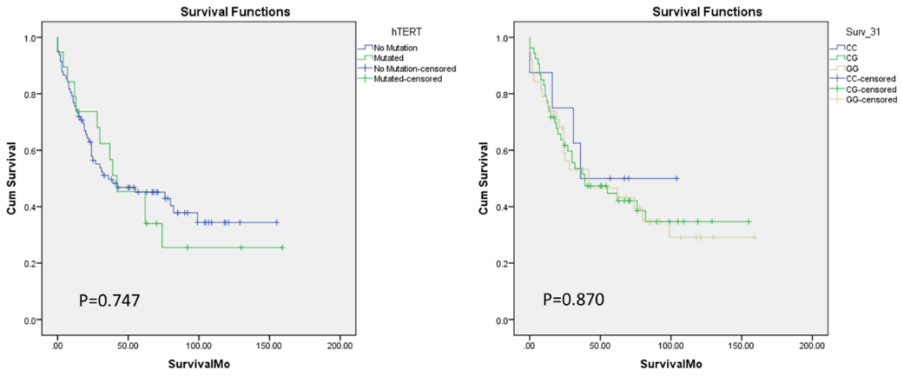


Figure 43. Cumulative survival based on Telomerase/ Survivin promoter status across all malignancies.

There was no difference in survival between $pTERT^{Mut/WT}$, as shown in the Kaplan-Meier plot in figure 43, χ^2 (1) = 0.104, p= 0.747. Average survival data for WT tumours include a mean survival time of 71.8 +/- 7.7 months (95% CI 56.6 – 86.9) and median survival time of 36.0 +/- 22.7 (95% CI 0.0 – 80.5). Patient with $pTERT^{Muts}$ had a mean survival time of 65.7 +/- 14.2 months (95% CI 37.9 – 93.5) and a corresponding median survival of 42.0 +/- 12.9 months (95% CI 16.7 – 67.3).

There was no difference in cumulative survival based on the Survivin promoter allele status, χ^2 (2) = 0.278, p= 0.870. Average survival data for CC homozygotes includes a

mean survival duration of 62.4 +/- 15.1 months (95% CI 32.7 – 92.0) with no median survival data fewer than 50% of these patients died. CG heterozygotes had a mean survival of 71.9 +/- 9.4 months (95% CI 53.5 – 90.2) and median survival of 39.0 +/- 16.2 months (95% CI 7.3 – 70.7). GG homozygotes had a mean survival of 69.5 +/- 11.1 months (95% CI 47.7 – 91.3) and median survival of 42.0 +/- 24.4 months (95% CI 0.0 – 89.9).

3.3.9.3 HCC+FL

Subgroup analyses of all HCC tumours (including FL-HCC) also found no associations between promoter status and cumulative survival, as can be seen in figure 44.

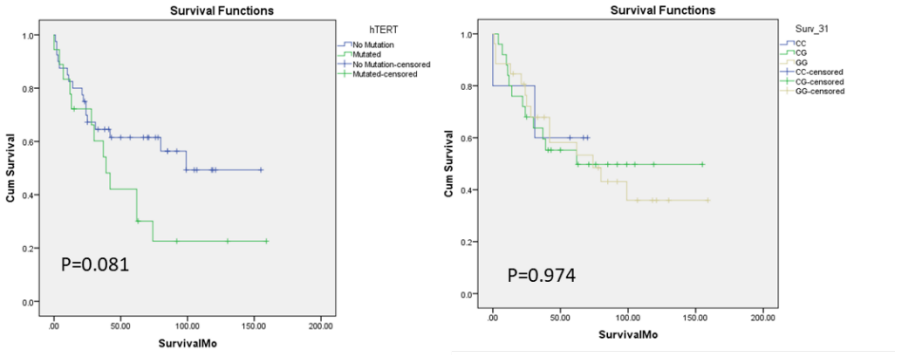


Figure 44. Cumulative survival based on Telomerase/ Survivin promoter status across all HCC subtypes.

There was no difference in survival between pTERT$^{Mut/WT}$, as shown in the Kaplan-Meier plot in figure 44, χ^2 (1) = 3.043, p= 0.081. Average survival data for WT tumours include a mean survival time of 93.8 +/- 11.1 months (95% CI 72.0 – 115.5) and no median survival data as >50% of patients survived. Patient with pTERT^{Muts} had a mean survival time of 61.5 +/- 13.9 months (95% CI 34.2 – 88.8) and a corresponding median survival of 39.0 +/- 8.0 months (95% CI 23.3 – 54.7). Cox regression analysis also failed to demonstrate any correlation between Survival and pTERT$^{Mut/WT}$ status.

There was no difference in cumulative survival based on the Survivin promoter allele status, χ^2 (2) = 0.053, p= 0.974. Average survival data for CC homozygotes includes a mean survival duration of 48.2 +/- 12.7 months (95% CI 23.3 – 73.1) with no median

survival data fewer than 50% of these patients died. CG heterozygotes had a mean survival of 89.2 +/- 13.7 months (95% CI 62.3 – 116.0) and no median survival data fewer than 50% of these patients died. GG homozygotes had a mean survival of 84.0 +/- 13.1 months (95% CI 68.4 – 105.2) and median survival of 74.0 +/- 26.7 months (95% CI 21.7 – 126.3.

3.3.9.4 HCC Only

Assessing associations between promoter status and survival in HCC (excluding FL-HCC) also yielded no significant associations, as seen in figure 45.

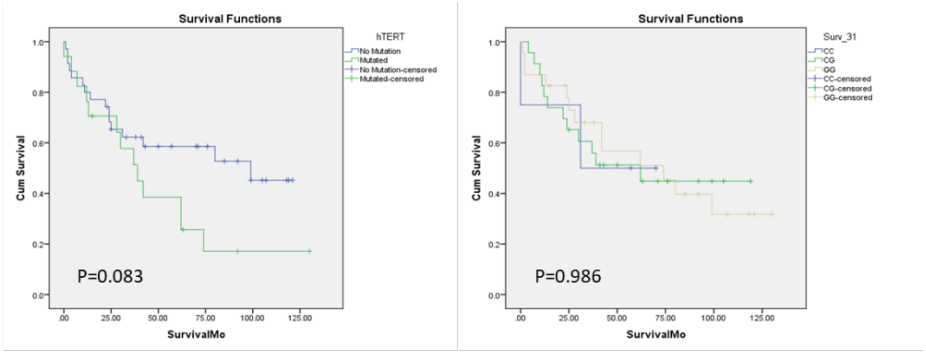


Figure 45. Cumulative survival based on Telomerase/ Survivin promoter status across traditional HCCs.

There was no difference in survival between pTERT$^{Mut/WT}$, as shown in the Kaplan-Meier plot in figure 45, χ^2 (1) = 3.006, p= 0.083. Average survival data for WT tumours include a mean survival time of 73.5 +/- 8.8 months (95% CI 56.2 – 90.8) and no median survival data as >50% of patients survived. Patient with pTERT^{Muts} had a mean survival time of 49.9 +/- 10.7 months (95% CI 29.0 – 70.9) and a corresponding median survival of 39.0 +/- 8.7 months (95% CI 21.9 – 56.7). Cox regression analysis also failed to demonstrate any correlation between Survival and pTERT$^{Mut/WT}$ status.

There was no difference in cumulative survival based on the Survivin promoter allele status, χ^2 (2) = 0.029, p= 0.986. Average survival data for CC homozygotes includes a mean survival duration of 42.8 +/- 14.7 months (95% CI 14.0 – 71.5) with no median survival data fewer than 50% of these patients died. CG heterozygotes had a mean

survival of 66.8 +/- 10.5 months (95% CI 46.3 – 87.3) and a median survival of 62.0 +/- 24.9 months (95% CI 13.1 – 110.9). GG homozygotes had a mean survival of 70.7 +/- 10.8 months (95% CI 49.4 – 91.9) and median survival of 74.0 +/- 25.5 months (95% CI 23.9 – 124.0).

3.3.9.5 Cholangiocarcinoma

There were no pTERT^{Muts} in CCA, but the survival curves for pSurv can be seen in figures 46 & 47. Figure 46 shows all CCAs analysed collectively and figure 47 is a subgroup analysis of all intrahepatic CCA.

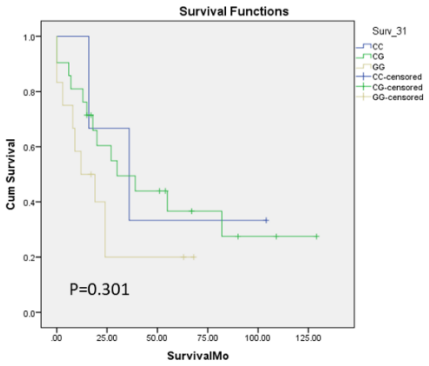


Figure 46. Cumulative survival based on Survivin promoter status across all CCAs.

There was no difference in cumulative survival based on the Survivin promoter allele status, χ^2 (2) = 2.403, p= 0.301. Average survival data for CC homozygotes includes a mean survival duration of 52.0 +/- 21.7 months (95% CI 9.4 – 94.6) with a median survival of 36.0 +/- 16.3 months (95% CI 4.0 – 68.0). CG heterozygotes had a mean survival of 56.3 +/- 11.8 months (95% CI 33.2 – 79.4) and a median survival of 30.0 +/- 13.2 months (95% CI 4.2 – 55.8). GG homozygotes had a mean survival of 23.0 +/- 7.2 months (95% CI 8.8 – 37.1) and median survival of 12.0 +/- 7.9 months (95% CI 0.0 – 27.4).

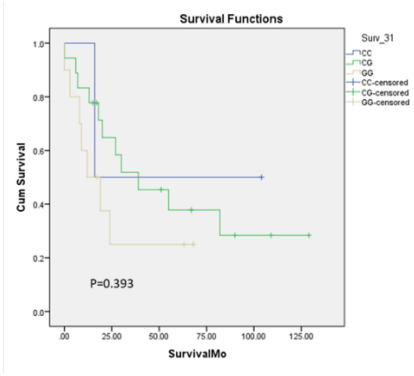


Figure 47. Cumulative survival based on Survivin promoter status across intrahepatic CCAs.

There was no difference in cumulative survival based on the Survivin promoter allele status, χ^2 (2) = 1.867, p= 0.393. Average survival data for CC homozygotes includes a mean survival duration of 60.0 +/- 31.3 months (95% CI 0.0 – 121.0) with no median survival as >50% of patients survived. CG heterozygotes had a mean survival of 58.6 +/- 12.5 months (95% CI 34.1 – 83.1) and a median survival of 39.0 +/- 17.0 months (95% CI 5.7 – 72.3). GG homozygotes had a mean survival of 25.6 +/- 8.5 months (95% CI 9.0 – 42.2) and median survival of 12.0 +/- 7.0 months (95% CI 0.0 – 25.7).

3.3.9.6 Mixed Tumour pTERT survival analysis

All seven of the mixed tumours were CG heterozygotes therefore assessing cumulative survival based on pSurv status was not possible. There was a single pTERT^{Muts} in the mixed tumour cohort, the only survivor, however the small size of the cohort meant that this was did not result in a significant result when considering cumulative survival, χ^2 (1) = 1.437, p= 0.231, figure 48.

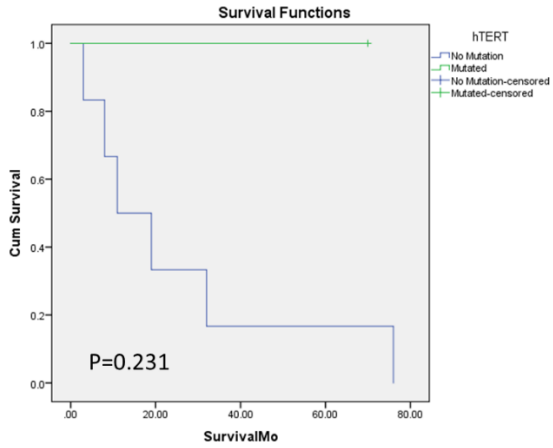


Figure 48. Cumulative survival based on the Telomerase promoter status in mixed HCC-CCA.

3.3.10 Discussion

There are no significant relationships between promoter status and cumulative survival for either pSurv SNPs or the pTERT^{Muts}. Even when tumour subgroups were analysed correlation between promoter status and cumulative survival was consistently absent in this small study cohort. Telomerase promoter mutations have been shown to be an HCC-lineage specific genetic alteration, and it appears that cumulative survival may be shorter in these patients both within the whole HCC cohort (p= 0.081) and when the FL-HCCs are excluded (p= 0.083). In a larger cohort of HCCs this relationship could be further clarified, which highlights the possibility of using genetic analysis to stratify patients into high, and low-risk groups for clinical follow up.

3.4 Promoter Sequences - Discussion

Amplifying DNA extracted from FFPE tissues presented significant technical challenges but with persistence and much refinement of the methods used ultimately yielded some very interesting results. Given that every hospital pathology department in the country, if not the world, uses FFPE tissue blocks for diagnostic purposes the methods used here are readily transferable to an enormous potential research resource. This could open up the entire research archive for assessment (not only of UTAA promoter sequences) giving a truly representative picture of promoter sequences, which

might only be limited by archival and storage capacity and storage logistics. However, there are some limitations when using poor quality DNA but in the absence of a frozen tissue biobank, these results demonstrate similar detection rates to those found in the published literature – such as the minor allele frequency of pSurv and the rate of $pTERT^{Muts}$ in HCCs.

3.4.1 Major Findings

The Telomerase promoter mutation results are the most interesting, and statistically significant findings in the sequencing results. There was consistent evidence of $pTERT^{Muts}$ being associated with male gender when all neoplasms ($p= 0.001$) and the malignancies ($p= 0.001$) were compared.

There was also considerable evidence of associations with $pTERT^{Muts}$ and HCC, and HCC-lineage, tumours in the whole cohort ($p= 0.002$) and in the malignant cohort ($p= 0.003$). Therefore, the putative associations with fibrosis ($p= 0.009$), cirrhosis ($p= 0.004$) in the whole cohort must be interpreted with some caution, as both fibrosis and cirrhosis are pre-cancerous conditions that cause HCCs to develop in the context of chronic cirrhosis.

Within the HCC-only cohort of tumours the male preponderance was also present ($p= 0.005$) as was a weak association with all-cause mortality ($p= 0.043$) and slightly more convincing association with vascular invasion ($p= 0.031$) and $pTERT^{Muts}$. However, when considering cumulative survival there was reduced median survival in $pTERT^{Muts}$ compared to $pTERT^{WT}$, but this lacked significance in both the classical HCCs ($p= 0.083$) and when combined with FL-HCCs ($p= 0.081$).

The Survivin promoter SNP was confirmed as a germline in nature given the 100% concordance between background liver, and tumour tissues. This was the limit of the major findings in the Survivin promoter sequence in this cohort.

3.4.2 Minor Findings

Significantly fewer cirrhosis-associated neoplasms were associated with the Survivin promoter GG homozygous allele across the whole study cohort, p= 0.042. However, this result was not significantly replicated in sub-analyses, including the malignant cohort, p= 0.078. An apparently lower rate of cirrhosis in GG homozygotes (p= 0.042) is of some interest when assessing the whole cohort. This could be because of the two main tumour groups (HCC & CCA) there was an equal number of CG/GG (n=23) alleles in the HCCs, whilst there were more heterozygotes (21) compared to GG homozygotes (12) in the CCAs.

When tumour size and patient age are considered, the pTERT^{Muts} status may be important in larger malignancies. Across the whole cohort pTERT^{Muts} correlate with larger tumours (p= 0.083) and advanced patient age (p= 0.065). However, then the benign Adenomas are excluded (of which 90% occur in young women in this study) pTERT^{Muts} lose their correlation with patient age (p= 0.204) but still appear to occur in (non-significantly) smaller malignant tumours, p= 0.089.

When considering pTERT^{Muts} in the malignant cohort, the associations between fibrosis (p= 0.01) and cirrhosis (p= 0.012) are weaker, which is not terribly surprising given that Adenomas occur in young women, rather than in the context of chronic inflammation. The weak association between pTERT^{Muts} and fibrosis in all HCCs (p= 0.044) becomes non-significant when the FL-HCCs – which are not associated with traditional risk factors for HCC development - are excluded (p= 0.075). This casts some doubt on the implication of these findings in this small study cohort. However, there is also a significant selection bias in these surgically managed tumours, which will certainly impact any potential impact of this data.

3.4.3 Commentary and Future work

There is evidence of a potential sampling bias with both inter-tumour and intra-tumoural heterogeneity in the expression of Telomerase promoter mutations. For patients 24 and 116, DNA extracted from different tumour tissue blocks gave different $pTERT^{Muts}$ results. This could either be due to true tumour heterogeneity, or a result an unsatisfactory lower limit of detection for assigning mutation/WT status in amplified DNA sequences. It is thought-provoking to consider whether $pTERT^{Muts}$ would also be present in metastatic tumours, which would require a refinement of the methodologies to allow sequencing from biopsy samples.

However, the pTERT mutation rate (31-32%) is within the range that has been previously reported at an international level (30-60%) but is lower than expected for the predominantly European, compared to Asian, population that has been studied here. Whether this is a true representation, or falsely low is difficult to state with any certainty. To further answer this question, the same techniques could be used on DNA taken from multiple tissue blocks, to fully characterise the cohort. Introducing a more sensitive amplification methods could also achieve this. This has been shown in Melanoma, where Telomerase promoter mutations are an independent prognostic risk factor, so perhaps increasing the study size will lead to greater clarity on the role $pTERT^{Muts}$ have in HCCs, (Griewank *et al.*, 2014).

Future work could include using alternative techniques, such as droplet digital PCR (which has recently become more accurate) for detecting point mutations. Detection rates for ddPCR have been quoted as being roughly double that of Sanger sequencing when detecting the Y373C FGFR3 mutation in bladder cancer (Borkowska *et al.*, 2019). Some work has been published in the pTERT field quoting a lower limit of detection of 0.17% when using ddPCR to assess malignant melanoma, albeit whilst using Sanger sequencing as the gold standard (McEvoy *et al.*, 2017).

What must be remembered in this cohort is the sampling bias of patients that are fit enough to undergo major abdominal surgery. Only 23 of the patients are cirrhotic, which is known to be a major driver in carcinogenesis, so caution should be exercised when interpreting the findings. Further work could include sequencing needle biopsy specimens, to ascertain whether this is technically feasible and yields meaningful results. This would allow a more comprehensive cohort of tumour samples to be sequenced, helping to clarify any possible relationships I have found.

The Survivin promoter work has, broadly, been less interesting than that of $pTERT^{Muts}$. Apart from the concordance between tumour and background liver samples, there are few correlations with clinical disease outcomes as in the pTERT analyses. Expanding the Telomerase-activation studies to include other activating events (DNA integration, ALT, etc) could highlight the role of Telomerase in PLCs. Techniques required for this would need fresh/frozen materials, but these findings in FFPE tissues is a solid foundation to build upon.

Critiquing the technical methods used would allow me to potentially improve the confidence of the sequencing results. It would potentially have been more scientifically rigorous to run a positive control for every PCR reaction, but given the use of no-template negative controls ensuring no contaminated samples, it was felt that this was adequate. However, previous work in the field (i.e., not using a known positive control) has found this approach satisfactory, and been adopted in completely different fields where genotyping by PCR is done (Radojevic-Skodric *et al.*, 2012; Jacquot *et al.*, 2019). As the number of samples processed simultaneously on a single gel (n=22) extracted using the same technique, samples acted as their own internal control, rather than relying on an exogenous/endogenous control run in the same reaction tube that may reduce the efficiency of the PCR (QIAGEN, 2020). As stated by QIAGEN on their website, negative controls are adequate to rule out any contaminations interfering with

the assessment of nucleic acids, whilst endogenous homologous internal controls will compete for reagents and reduce the efficiency of the reactions.

4 Survivin Immunohistochemistry

4.1.1 Introduction

This chapter describes my immunohistochemistry findings to define Survivin expression in a range of tumour types. Ensuring the proteomic detection, and quantification, of tumour-associated antigens required rigorous optimisation to ensure an accurate (and meaningful) interpretation of the data was possible. Assessing the expression of UTAA in PLCs was reliant on commercially available antibodies that have been extensively used in peer-reviewed publications. The Survivin antibody ultimately chosen for this research project has been used many times in the published literature (219 at the time of selection, 228 at the time of writing, including in FFPE studies) giving weight to the reliability of the results (Cell Signalling Technology, 2020).

4.1.2 Survivin IHC Optimisation

Almost without exception Survivin 71G4B7 Rabbit monoclonal antibody (Cell Signalling) has consistently used nuclear staining in tonsillar tissue for a positive control. Published literature and online resources such as the Human Protein Atlas (HPA) confirm significant expression of nuclear Survivin in tonsil tissues, which made the initial optimisation steps straightforward (Thul *et al.*, 2017; Cell Signalling Technology, 2020). Optimisation, as outlined in figure 49, found the best dilution of primary antibody.

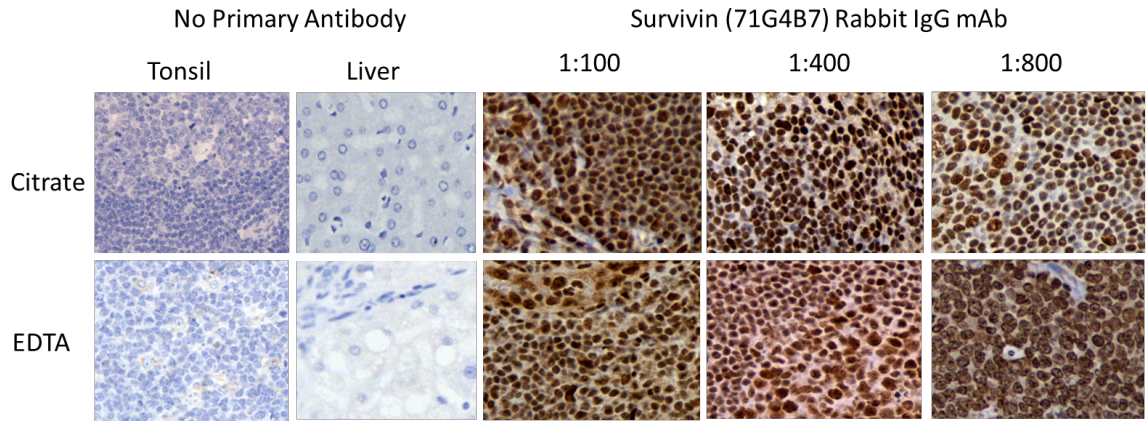


Figure 49. Optimisation steps for the Survivin primary antibody. Images shown at 200X magnification.

With citrate buffer for antigen retrieval and a 1:400 dilution of antibody with overnight incubation at 4 Celsius selected as giving the best balance of strong positive staining with minimal background 'noise.' Figure 49 highlights the quality of this antibody as there is no expression in background liver tissues. Experiments were repeated in triplicate, on different days, to ensure reproducibility and reliability of the protocol. Source material (Tonsil) was used in every batch of IHC as a positive control, and if the control tissue block was being diminished, a separate tissue block of tonsil was run in parallel to ensure consistency in nuclear staining between positive controls.

4.1.3 QuPath Assessment of Tissues: Survivin

The perfect artificial intelligence software would simultaneously analyse tissues, undertake quantification and use machine learning to constantly improve accuracy. This would involve QuPath assessing the morphological features observed in a specific tumour type and would require both exploratory and validation cohorts. However, this would have required some degree of un-blinding during this - hypothetical - optimisation process which, although desirable, was not feasible whilst working within the ethical constraints of this project.

As outlined above: I, the researcher, am to be blinded to the tissues being studied. This meant the creation of classifiers (a method of training the software to recognise cell

types - more on this later) somewhat difficult. Training software to recognise HCCs and CCAs simultaneously as ‘tumour’ resulted in poor quality results when compared to an expert commentator’s assessment. As both HCCs and CCAs demonstrate significantly different cellular morphology it is unsurprising that an object classifier designed to differentiate between cells (i.e., tumour versus benign versus inflammatory cell) based on cellular morphology struggled in this combined cohort. Furthermore, when more than 10 images (each image-file being around 3-4 gigabytes in size) were open simultaneously with the same classifier the software regularly froze, or crashed, which made for a slow and frustrating process.

As a result of both the ethical and logistical issues during optimisation, the decision to create individual classifiers for each slide was made. The classifier is able to detect both the nuclear haematoxylin (blue) and the chromogen (in this case a brown colour) using a process called colour deconvolution which the human eye is unable to do. QuPath’s default is to use DAB/Haematoxylin for classifiers, meaning this process is standardised, and therefore reproducible. This meant the quality of the data was reliable and also compared very favourably with an expert commentator's assessment of staining. It also meant the computing power available was not regularly overloaded when assessing protein levels, which sped up the quantification steps considerably. This case-by-case analysis relies on accurate assessment of the tissue samples being studied, with an inherent bias that should be acknowledged when interpreting the results.

Whole tissue slides were assessed for the proportion and intensity of staining positivity in both tumour and background liver samples. Selective tissues can be seen in the images below, comparing the raw image files (bottom row) and the colour coded (top row) data post-processing in figure 50.

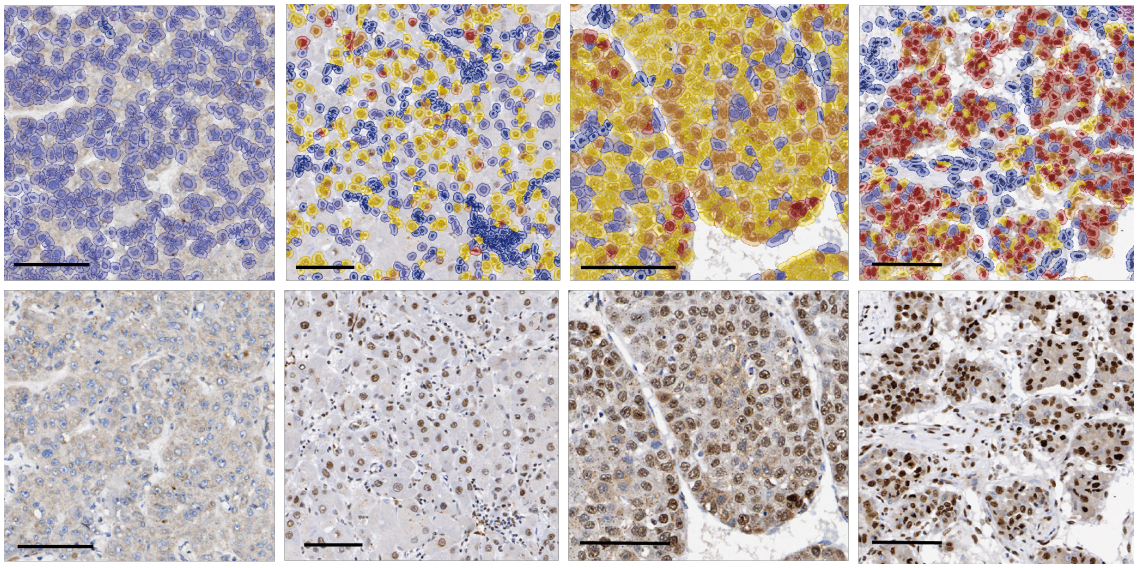


Figure 50. HCC tumours with varying degrees of nuclear positivity for Survivin. Scale bar: 100 microns.

The colour scheme is as follows: negative (blue), + (yellow), ++ (orange) and +++ (red) as the intensity of the nuclei staining progresses (lower images) with warmer colours (upper images) predominating from left to right in figures 50 & 51. Both negatively staining tumour nuclei and stromal cells are shown by subtly different shades of blue.

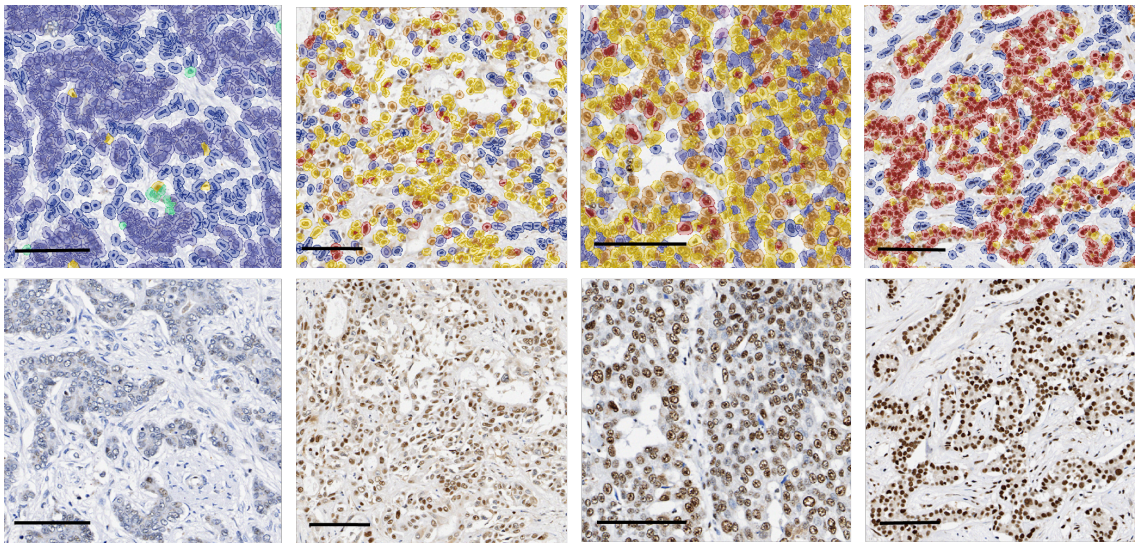


Figure 51 CCA tumours with varying degrees of nuclear positivity for Survivin. Scale bar: 100 microns.

For CCA, the differences in staining intensity can be seen in figure 51. In both figures 50 and 51 there is evidence of clear nuclear staining with minimal stromal, or cytoplasmic, staining.

4.1.4 IHC Results: Survivin

Survivin immunohistochemistry staining, allowing levels to be assessed, was achieved in 98.2% (109/111) of tumours and in 85.6% (95/111) of the background tissues. The two tumour samples that were not able to be assessed were both extensively necrotic and therefore did not counterstain with nuclear haematoxylin, thus rendering them un-assessable. Of the 16 cases where background liver tissue was unable to be assessed 13 (11.7%) were because there was no background tissue available for the study, and the remaining three (2.7%) were extensively necrotic and also lacked haematoxylin counterstaining. Internal positive controls (lymphocytes) were present and all tissues and demonstrated, stained positively, and were accounted for when the project classifier was being trained.

Average expression of Survivin, quantified using both the Allred (0, 2 – 8) and H-scores (0 – 300), can be seen in table 40. Comparisons between paired tumour and background staining, and the statistical significance of these differences, can also be seen in this table.

All Neoplasms	**H-Score**		**Allred Score**	
	Tumour	**Background**	**Tumour**	**Background**
Mean +/- S.D.	82.9 +/- 62.6	15.1 +/- 1.4	5.2 +/- 1.4	3.3 +/- 1.0
Min - Max (Range)	0.5 - 239.8 (239.3)	0.0 - 148.7 (148.7)	2 – 8 (6)	2 – 7 (5)
Median	63.9	3.6	5.0	3.0
Test Statistic	Z= -8.1, p= 4.8 E-16		t(92) = 13.1, p= 9.2E-23.	

Table 40. Average tumour and background liver Survivin levels using the H-score and Allred score.

Table 40 shows significantly more Survivin in tumour compared with paired background tissue-staining scores across all samples by both quantification methods. The median values (for the non-normally distributed difference in H-Score) and paired mean values (using the normally distributed difference in Allred score) both confirmed significantly more Survivin expression in tumour, compared to paired background liver

tissues (p= 4.77E-16 and p= 9.19E-23 respectively). It is noteworthy at this stage to emphasise that the differences in Allred scores are normally distributed, and that the tumour protein levels have greater statistical significance. In analyses comparing protein expression in tumour compared to background liver tissues the overall differences in expression were visualised, thus determining the statistical test subsequently selected. The Wilcoxon signed-rank test does not assume normality, as opposed to the Paired-Samples T-test, the most appropriate statistical test was used depending on the original data. The test statistic used can be simply ascertained when consulting the tables below: the Z-score (i.e., Z=) denotes the Wilcoxon signed-rank test whilst the Paired-Samples T-test is shown by (t(degrees of freedom)=).

Given the germline nature of the Survivin promoter *rs9904341* I investigated for potential differences between the intensity of staining in tumour/background tissues with CC, CG and GG Survivin alleles. No significant differences were found figure 52 demonstrates the non-significant differences across the three alleles.

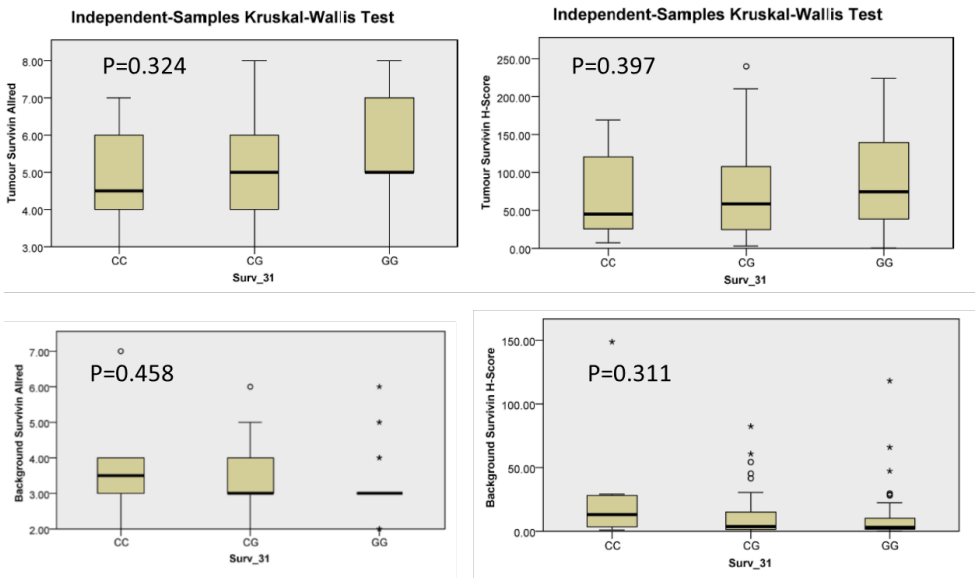


Figure 52. No allele preferentially expressed more Survivin when assessing all neoplasms, using both the Allred and H-Scores.

Also, there was a significant difference in Survivin staining between tumour types when considering both the neoplastic tissues (figure 53, top row) and background livers (bottom row).

Figure 53 outlines differences in Survivin expression by tumour sub-type.

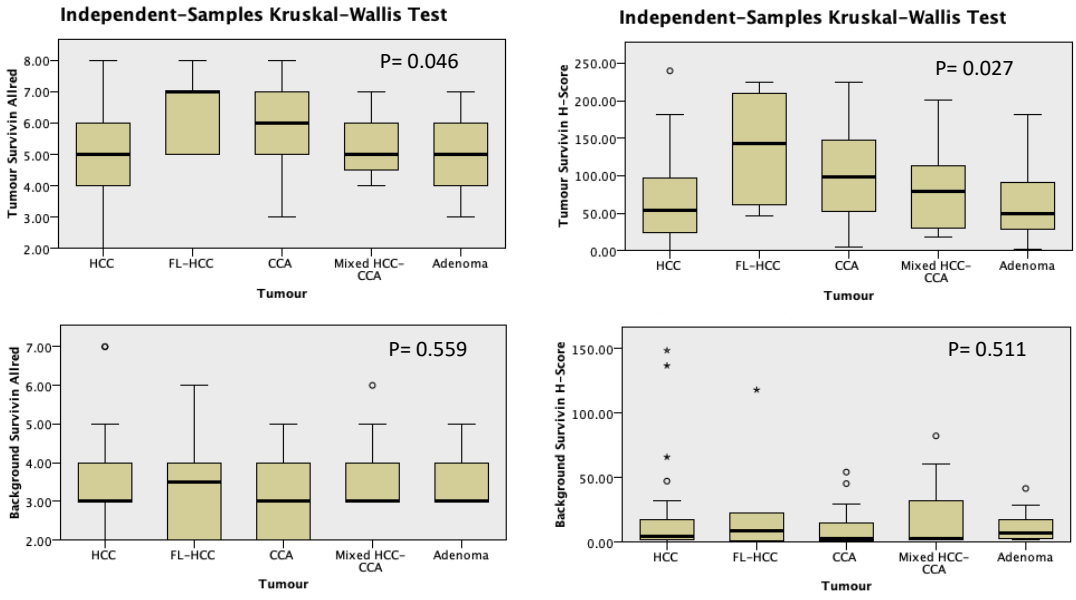


Figure 53. Differential expression of Survivin by tumour type.

The H-scores (χ^2 (4) = 10.923, p= 0.027) and the Allred scores (χ^2 (4) = 9.701, p= 0.046) varied between tumour types with average values shown in tables 41 and 42.

H-Score	HCC	FL-HCC	CCA	Mixed HCC-CAA	Adenoma
Mean +/- S.D.	68.6 +/- 57.4	138.0 +/- 73.8	100.7 +/- 62.5	83.5 +/- 65.8	60.3 +/- 54.8
Median	53.1	143.2	98.1	79.3	54.8
Mean Rank	47.9	79.7	64.6	55.0	43.0

Table 41 Average H-score values (scale 0 – 300) for Survivin expression across tumour types.

Table A shows the highest level of detected Survivin was found in FL-HCCs with the lowest in Adenomas. Uncorrected pairwise comparisons of the H-scores are significantly different between FL-HCC and Adenoma (p= 0.025), HCC and CCA (p= 0.016) and HCC and FL-HCCs (p= 0.020). However no pairwise differences differed significantly when adjusted for multiple analyses.

Allred Score	HCC	FL-HCC	CCA	Mixed HCC-CAA	Adenoma
Mean +/- S.D.	5.0 +/- 1.3	6.5 +/- 1.2	5.6 +/- 1.4	5.3 +/- 1.1	4.9 +/- 1.4
Median	5.0	7.0	6.0	5.0	5.0
Mean Rank	48.4	81.7	62.5	54.9	46.7

Table 42. Average Allred scores (0, 2 - 8) for Survivin expression across tumour types.

When the Allred score was used the highest levels were also detected in FL-HCCs (as shown in table 42) and the lowest in Adenomas. Similar to the H-score analysis, uncorrected pairwise comparisons for Allred scores are also significantly different between FL-HCC and Adenoma (p= 0.028), HCC and CCA (p= 0.036) and HCC and FL-HCC (p= 0.012). Similar to the H-score findings, once the significance was adjusted for multiple analyses, none of these differences were statistically significant.

In summary I have found that there is a significant difference in tumour/ background liver Survivin expression (table 40) with differential expression of Survivin depending on the tumour type (figure 53). However, within this small study cohort the significance of pairwise comparisons is not certain when corrected for multiple analyses, meaning further work would help clarify whether this is a true relationship, or not.

4.1.4.1 Clinical Correlates with Tumour Survivin Expression

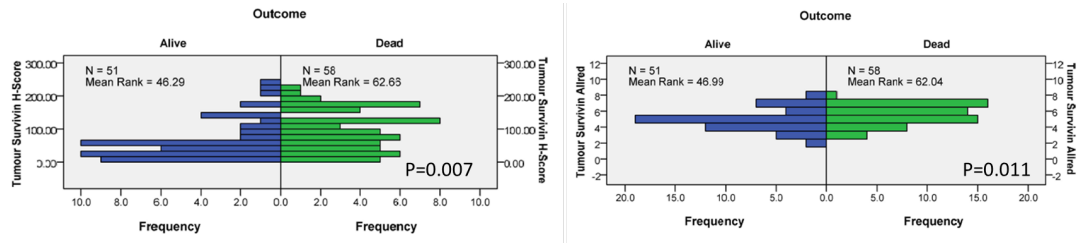
Survivin levels across the whole cohort were correlated with clinical measures. Table 43 outlines protein expression differences between groups with P-values representing the level of significance of differences between the nominal outcome variable (Gender: M/F. Outcome: Dead/Alive, etc) and whether the tumour/ background Survivin quantification scores have a statistically significant difference between these groups. There is no data in table 43 about whether these differences are positive or negatively correlated, as this will be addressed subsequently.

No statistically significant association between variation in Survivin expression in background liver tissue and clinical correlates was found. However, tumour Survivin expression was found to correlate with several clinical characteristics.

All Neoplasms	Tumour Survivin		Background Survivin	
	H-Score	Allred Score	H-Score	Allred Score
Gender	0.591	0.568	0.964	0.694
Outcome	**0.007**	**0.011**	0.433	0.360
Fibrosis	0.231	0.272	0.824	0.812
Cirrhosis	0.053	0.061	0.923	0.742
Viral Hepatitis	0.213	0.267	1.000	0.906
Metabolic Risk Factors	0.575	0.933	0.748	0.974
Genetic Liver Disease	0.834	0.996	0.228	0.594

Table 43. The distribution of Survivin staining across all neoplasms, based on clinical measures.

Survivin expression appears to be differentially expressed between patients who have gone on to die, compared to those still alive. There is no significant difference in tumour Survivin expression (quantified using both the Allred and H-score) between: genders, fibrosis/cirrhosis derived tumours, and did not differ based on any further underlying inflammatory condition (viral hepatitis, metabolic or genetic diseases). Importantly: there were no significant relationships found between Survivin expression in background liver tissues and any clinical parameter measured. However, some caution must be exercised when comparing malignant characteristics (vascular invasion, margin positivity, etc) when the benign Adenomas are included – despite these being coded as missing data in SPSS – therefore these results will be further clarified below, figure 54.



Outcome		H-Score			Allred		
Group	**N**	**Mean Rank**	**Mean +/- S.D.**	**Median**	**Mean Rank**	**Mean +/- S.D.**	**Median**
Alive	51	46.29	67.04 +/- 61.27	50.68	46.99	4.92 +/- 1.41	5.00
Dead	58	62.66	96.91 +/- 60.84	95.73	62.04	5.57 +/- 1.27	6.00
Mann-Whitney U		1923			1887.5		
P-Value		**0.007**			**0.011**		

Figure 54. Higher Survivin levels are associated with all-cause mortality.

As can be seen from figure 54, using both the Allred and H-Scores, the mean ranks of Survivin were higher in tumour of those who died (62.04 and 62.66 respectively) compared to those alive (46.99 and 46.29, respectively). The level of statistical significance varies depending on the method of quantification used (p= 0.007 for the H-Score and p= 0.011 for the Allred score).

Scale variables such as tumour size in mm, and patient age at the time of operation were compared with the intensity of staining measures, as outlined in table 44.

All Neoplasms		Tumour Survivin		Background Survivin	
		H Score	Allred	H Score	Allred
Age	Correlation Coefficient	0.160	0.113	-0.101	-0.16
	P Value	0.097	0.243	0.330	0.122
Tumour Size	Correlation Coefficient	0.171	0.170	0.054	-0.031
	P Value	0.075	0.077	0.601	0.780

Table 44. There is no correlation between tumour/background Survivin levels and the patient age (years) or the tumour size (mm).

There are no significant correlations between age or tumour size and Survivin in tumours or background liver tissues, but there is a suggestion of a positive correlation between tumour size and the intensity of staining (correlation coefficients ~ 0.17) and with significance P~0.076 reflected in similar values from both methods of recording tumour intensity staining. Patient age and tumour size are compared to staining intensity using Spearman's rank test with 2-sided P-values with the level of significance set at 0.05.

Continued use of both quantification methods makes for a somewhat clunky and repetitive analysis. Given that the Allred scores are easier to visualise, interpret, uses a smaller scale, appears to be more representative of the study cohort and have been used in other published works for differentiating between high/low protein expressors this method will be used for the remainder of the analysis. Stratifying tumours by high/low protein expression based on their Allred score has been reported in breast cancers. It appears to be a suitable way of conveying protein expression by combining both the

intensity of staining, as well as the percent of tumour that positively stain (Ellis *et al.*, 2010; Campbell *et al.*, 2016). In both of these published papers an Allred score of 6-8 was deemed 'high expression' when assessing the Oestrogen/Progesterone receptor expression when used to prognosticate, or otherwise stratify patients for offering an alternative chemotherapy regimen. However, two methods differentiating between groups was undertaken for completeness, as shown in figure 55.

Assigning a threshold for a Survivin^{High} score of 5-8 (left image) or 6-8 (right image) helps to differentiate survival characteristics based on Survivin expression in tumours as shown in the Kaplan-Meier survival curves in figure 55.

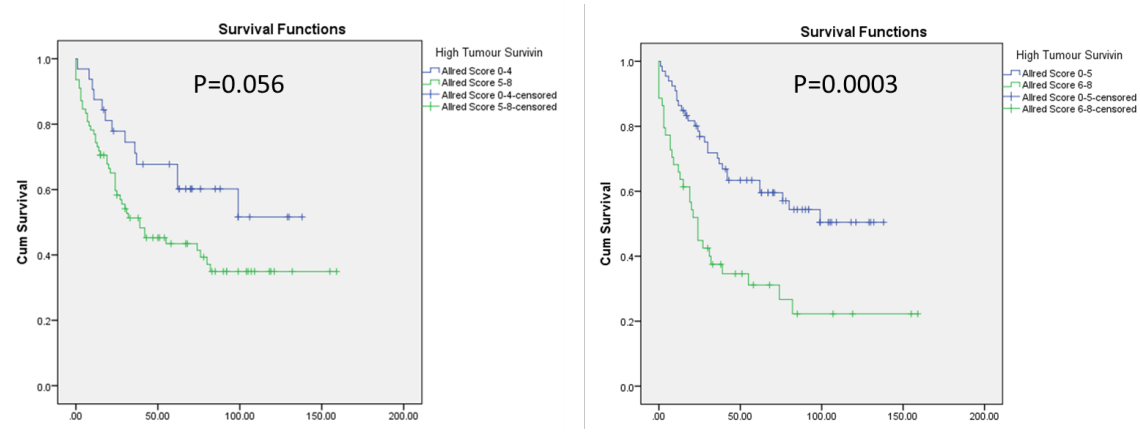


Figure 55. Tumours that express high levels of Survivin are associated with a reduced cumulative survival.

With high levels of tumour Survivin (i.e., Surv^{High} = Allred score 6 – 8) demonstrating a clearer, more statistically significant, reduction in cumulative survival, χ^2 (1) = 13.013, p= 0.003. Using this method, patients with Survivin^{Low}-expressing tumours had a longer median survival (62.0 +/- 17.0 months, 95% CI 28.6 – 95.4) compared with Survivin^{High}-expressing neoplasms (24.0 +/- 3.2 months, 95% CI 17.7 – 30.3).

Using the alternative (but not used elsewhere in the literature) threshold for tumour positivity (e.g., 0-4 low, and 5-8 high) gave a non-significant reduction in overall survival in tumours that express more Survivin, p= 0.056. Therefore, the higher cut off value for expression (Allred low scores 0-5 and high 6-8) was used for all further

tumour subgroup analyses. This approach was supported both by my data and the literature, table 45, (Ellis *et al.*, 2010; Campbell *et al.*, 2016).

All Neoplasms: Survivin		**N**	**Surv^{Low} % (n)**	**Surv^{High} % (n)**	**P-Value**
Gender	Female	45	55.6 (25)	44.4 (20)	0.437
	Male	65	63.1 (41)	36.9 (24)	
Outcome	Alive	51	74.5 (38)	25.5 (13)	**0.006**
	Dead	59	47.5 (28)	52.5 (31)	
Fibrosis	-	66	59.1 (39)	40.9 (27)	0.841
	+	43	62.8 (27)	37.2 (16)	
Cirrhosis	-	87	56.3 (49)	43.7 (38)	0.090
	+	22	77.3 (17)	22.7 (5)	
Viral Status	-	95	58.9 (56)	41.1 (39)	0.778
	+	15	60.0 (10)	40.0 (5)	
Genetic Liver Disease	-	100	60.0 (60)	40.0 (40)	1.00
	+	10	60.0 (6)	40.0 (4)	
Tumour	HCC	52	73.1 (38)	26.9 (14)	**0.032**
	FL-HCC	6	33.3 (2)	66.7 (4)	
	CCA	35	42.9 (15)	57.1 (20)	
	Mixed	7	57.1 (4)	42.9 (3)	
	Adenoma	10	70.0 (7)	30.0 (3)	
pSurv Allele	CC	8	75.0 (6)	25.0 (2)	0.352
	CG	55	65.5 (36)	34.5 (19)	
	GG	45	53.3 (24)	46.7 (21)	

Table 45. High tumour Survivin and clinical correlations.

High tumour Survivin is correlated with all-cause mortality, being detected in 52.5% (31/59) of those who have died compared with 25.5% (13/51) of those still alive, p= 0.006. Survivin^{High} was expressed to varying degrees depending on the tumour type, χ^2 (4) = 10.209, p= 0.032. The majority of HCCs preferentially expressed Survivin^{Low} with 73.1% (38/52) falling into this category, whilst the majority of CCAs (57.1%, 20/35) had Survivin^{High}. However as comparing malignant traits when including benign

tumours (Adenomas) within a cohort this may lead to some erroneous data being produced, these characteristics will be more fully assessed below.

More non-cirrhotic-derived tumours (43.7%, 38/87) expressed Survivin^{High} levels compared with tumours from cirrhotic patients (22.7%, 5/22), albeit to a non-significant extent, p= 0.090. Higher Survivin expression did not significantly correlate with gender, fibrosis, viral hepatitis, metabolic liver disease, genetic liver disease or any of the Survivin promoter variants, or subgroups of these variants. Teasing out the role of high tumour Survivin will be examined in the tumour subtype sections that follow.

4.1.5 Tumour Subgroup Analysis

As mentioned above, given the heterogeneous nature of the tumours being studied includes both benign and malignant neoplasms as well as clinical measures particular to certain tumours (e.g., perineural invasion in CCA, no malignant characteristics in Adenomas) subgroup analysis was undertaken to fully understand the study cohort.

4.1.5.1 All Malignancies

The clinical presentation, management and disease-course of primary liver malignancies is clearly very different from that of benign hepatic neoplasms. As such it is possible to compare malignant characteristics (tumour size, tumour stage, vascular invasion, etc) between tumour types, as well as overall survival data, further highlighting differences in Survivin expression and disease characteristics.

4.1.5.2 Clinicopathological Features

Reassuringly there was a highly significant difference between tumour and background Survivin levels, as shown in table 46.

All Malignancies	Allred Score	
	Tumour	Background Liver
Mean +/- S.D.	5.3 +/- 1.3	3.3 +/- 1.1
Min – Max (range)	2 – 8 (6)	2 – 7 (5)
Median	5.0	3.0
Test Statistics	t(82) = 12.9, p= 1.8E-21	

Table 46. Average Survivin expression in the malignant cohort.

Differences in mean Allred scores between tumour (5.3) versus background liver (3.3) were normally distributed (t(82)= 12.9, p= 1.8E-21), highlighting the highly significant nature of this relationship.

Survivin expression, based on tumour type, is shown in figure 56.

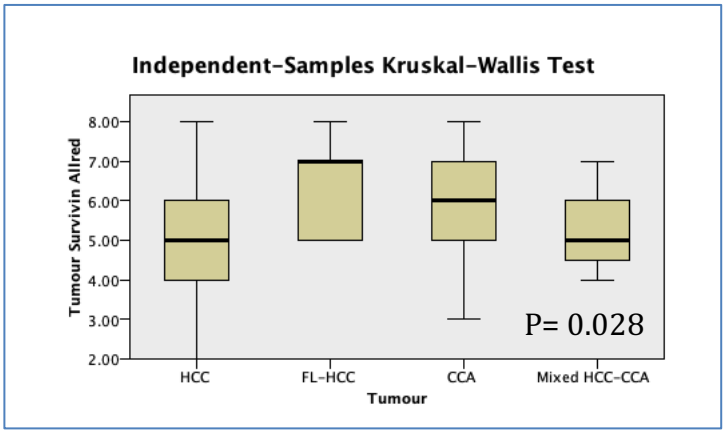


Figure 56 Differential Survivin expression by tumour type in malignancies.

Survivin was differentially expressed across malignant tumour types with FL-HCCs expressing the most (mean rank 73.7, median 7.0), followed by CCAs (56.2, median 6.0), Mixed tumours (49.0, median 5.0) and finally HCCs (43.1, median 5.0), χ^2 (3) = 9.079, p= 0.028, figure 56.

Allred Score: All Malignancies	**Correlation Coefficient (rho)**	**P-Value**
Patient Age	0.087	0.393
Tumour Size	0.201	**0.046**

Table 47. Across all malignancies, higher Survivin levels may be positively correlated with larger tumours.

When assessing tumour size and differential staining, it appears there is a weakly significant positive correlation (rho = 0.201, p= 0.046) between tumour size and Allred score. With benign tumours excluded it appears that larger tumours grow by evading apoptosis, table 47. There was no correlation between patient age and Survivin expression, p= 0.393. Comparing clinical characteristics in the malignancies is shown in table 48.

All Malignancies	**Tumour Allred Score Distribution**
Outcome (Dead vs Alive)	**P= 0.016**
Margin	P= 0.056
Cirrhosis	**P= 0.039**
Stage I & II vs. III & IV	**P= 0.047**
Vascular Invasion	**P= 0.008**

Table 48. Survivin distribution across all malignancies, based on clinic-pathological characteristics.

In the malignant cohort there is a highly significant relationship between tumour Survivin in tumours with vascular invasion (p= 0.008), as well as there being some role in overall outcome, p= 0.016. There is also a significant difference between those with liver cirrhosis (p= 0.039 and a marginally significant difference in Survivin expression when early (I & II) and late (III & IV) stage tumours, p= 0.047. Tumour Survivin expression did not differ significantly between R0 and R1 resection specimens, p= 0.056.

As shown in table 48 another property that is significantly different between groups is the vascular invasion of tumour. These differences are shown graphically, with more data, in figure 57.

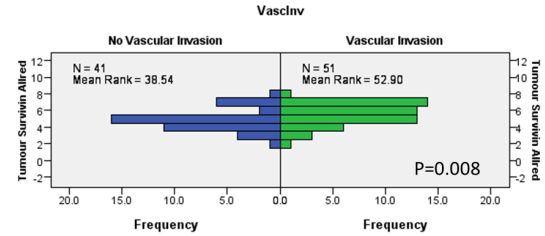


Figure 57. Higher Survivin levels are associated with vascular invasion of tumour samples.

Tumours with vascular invasion (VI^+) had a greater Allred score (mean rank of 52.9) compared to VI^- malignancies (mean rank of 38.5, U=1372, p= 0.008). Median Survivin values for VI^+ tumours was also greater (6.0) than in VI^- tumours (5.0). Both of these metrics highlight the invasive nature of Survivin expressing tumours and how greater Survivin expression can correlate with adverse histopathological features in this study cohort.

Differences in Survivin staining in early versus late-stage disease is of borderline significance with mean ranks lower in early (41.7) compared to late stage (53.5) disease, U= 1012, p=0.047, as shown in figure 58.

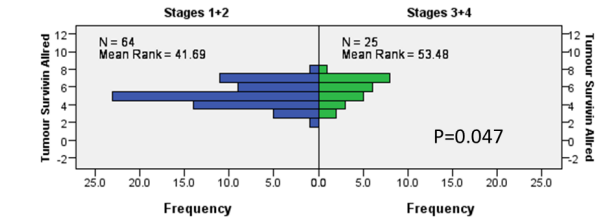


Figure 58. Higher Survivin levels may be associated with more advanced stage disease.

Median Survivin values for early-stage disease (5.0) was also lower than those found in more advanced tumours (6.0), implying this is perhaps a true representation of these average values.

As mentioned above, it was somewhat surprising to find higher levels of Survivin in tumours from non-cirrhotic, compared with cirrhosis-derived tumours when all malignant neoplasms are assessed, as shown in figure 59, p= 0.039.

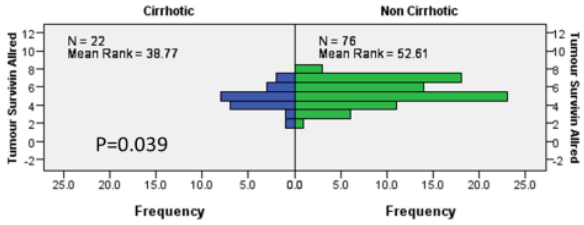


Figure 59. Across all malignancies, higher Survivin levels are significantly associated with tumours that arise in non-cirrhotic livers.

Surgically resected cirrhosis-derived liver tumours express less Survivin, as quantified with the Allred score, (mean rank 38.77) compared with non-cirrhosis-derived tumours (mean rank 52.61), U= 600, p= 0.039. However, the median values for both being 5.0, highlighting the borderline significance of this finding.

Furthermore, the study cohort is not representative of the majority of patients with liver cancer, meaning these findings need to be interpreted with caution owing to this selection bias. The asymmetric distribution of tumours arising on a background of cirrhosis (n=22, mostly *classical* HCCs) compared to those tumours arising from non-cirrhotic livers (n=76, almost all CCAs) will confound this finding. As I have shown above, CCAs express significantly more Survivin than HCCs so the significance of cirrhosis/ low-expressing tumours correlating is of perhaps somewhat limited value.

4.1.5.3 Overall Survival based on Survivin Expression

There remains a weakly significant difference in Survivin in tumours when assessing all-cause mortality, as shown in figure 60, p= 0.016.

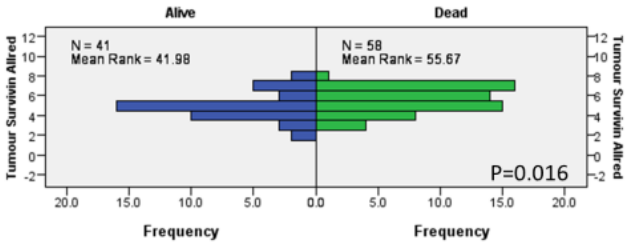


Figure 60. Across all malignancies, higher Survivin levels are significantly associated with all-cause mortality.

The Allred scores in malignant tumours from patients who have gone on to die is higher (mean rank 55.67) than in tumours from patients who are still alive (mean rank

41.98, U= 1518), p= 0.016. This is also reflected in the median values of those who died (6.0) compared with the alive patients (5.0).

Assessments of cumulative survival in Survivin^{High} malignancies is shown in the Kaplan Meier survival curve in figure 61, χ^2 (1) = 11.321, p= 0.001.

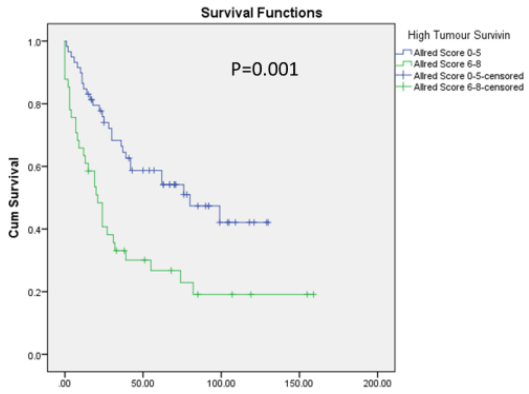


Figure 61. High Survivin expressing malignancies have a reduced cumulative survival.

Median survival in Survivin^{Low} expressing malignancies was 80.0 +/- 25.8 months (95% CI 29.4 – 130.6) compared to the shorter survival observed in Survivin^{High} expressing tumours 39.0 +/- 12.6 months (95% CI 14.2 – 63.8), p= 0.001.

4.1.5.4 Dichotomised Survivin Expression and clinical features.

Table 49 outlines how high tumour Survivin is associated with the adverse clinical measures of all-cause mortality, tumour stage and vascular invasion. As shown below in table 49, high tumour Survivin was more common in tumours from patients who subsequently died (31/59, 52.5%) compared with those still alive (10/41, 24.4%) p= 0.007. Over half of tumours (28/51, 54.9%) that invaded the vasculature had high tumour Survivin compared with 21.4% (9/42) in non-invasive tumours, indicating the association with this malignant characteristic, p= 0.001. Survivin^{High} is also present in more advanced tumours (stages III & IV), being detected in 60.0% (15/25) of late stage, compared with 32.3% (21/65) of early-stage disease, p= 0.029.

Malignancy - Survivin		N	Survivin^{Low} % (n)	Survivin^{High} % (n)	P-value
Outcome	**Alive**	41	75.5 (31)	24.4 (10)	**0.007**
	Dead	59	47.5 (28)	52.5 (31)	
Margin	**R0**	58	67.2 (39)	32.8 (19)	0.062
	R1	40	47.5 (19)	52.5 (21)	
Fibrosis	**-**	59	57.6 (34)	42.4 (25)	0.680
	+	40	62.5 (25)	37.5 (15)	
Cirrhosis	**-**	77	54.5 (42)	45.5 (35)	0.084
	+	22	77.3 (17)	22.7 (5)	
Tumour Stage	**I & II**	65	67.7 (44)	32.3 (21)	**0.029**
	III & IV	25	40 (10)	60 (15)	
Tumour Stage	**I**	30	83.3 (25)	16.7 (5)	**0.007**
	II	35	54.3 (19)	45.7 (16)	
	III	19	36.8 (7)	63.2 (12)	
	IV	6	50 (3)	50 (3)	
Grade	**Well**	11	72.7 (8)	27.3 (3)	0.716
	Mild/ Mod	65	58.5 (38)	41.5 (27)	
	Poor	17	58.8 (10)	41.2 (7)	
Vascular Invasion	**-**	42	78.6 (33)	21.4 (9)	**0.001**
	+	51	45.1 (23)	54.9 (28)	
Tumour	**HCC**	52	73.1 (38)	26.9 (14)	**0.018**
	FL-HCC	6	33.3 (2)	66.7 (4)	
	CCA	35	42.9 (15)	57.1 (20)	
	Mixed	7	57.1 (4)	42.9 (3)	
pSurv Allele	**CC**	8	75.0 (6)	25.0 (2)	0.228
	CG	52	65.2 (34)	34.6 (18)	
	GG	38	50 .0 (19)	50.0 (19)	

Table 49. High tumour Survivin correlates with the clinic-pathological measures.

When further characterising individual tumour stages, Survivin^{High} was found at increased levels in more advanced tumour stages with only 16.7% (5/30) of stage I, 45.7% (16/35) of stage II, 63.2% (12/19) of stage III and 50.0% (3/6) of stage IV, expressing high levels of tumour Survivin, χ^2 (3) = 11.777, p= 0.007. Also, Survivin^{High}

expression was significantly different between tumour types as only 34.6% (18/52) of HCCs compared with 57.1% (20/35) of CCAs highly expressed Survivin, χ^2 (3) = 9.674, p= 0.018.

There is a non-significant increase in tumour Survivin detected in tumours from non-cirrhotic (35/77, 45.5%) compared with cirrhotic livers (5/22, 22.7%) p= 0.084. Fewer of the R0 resections (19/58, 32.8%) expressed high tumour Survivin levels, compared with R1 resections (21/40, 52.5%, p= 0.062). There is no correlation between high tumour Survivin and tumour grade (p= 0.716), fibrosis (p= 0.680) or cirrhosis (p= 0.084) and no association with increased levels of Survivin and any particular *rs9904341* allele, p= 0.228.

Survivin^{High} may be a useful adjunct in determining higher-risk liver tumours as adversely associated clinical measures (vascular invasion, advanced tumour stage and highlighting patients that are at an increased risk of early death) are significantly associated with these characteristics in this relatively small malignant cohort.

4.1.5.5 HCC

In HCCs, as with all neoplasms I have studied, there is a significant increase in Survivin expression in tumours compared to background liver tissues, as shown in table 50.

HCC Allred Score	Tumour	Background Liver
Mean +/- S.D.	5.1 +/- 1.3	3.3 +/- 1.1
Min – Max (range)	2 – 8 (6)	2 – 7 (5)
Median	5.0	3.0
Test Statistic	t(42) = 8.8, p= 3.8E-11	

Table 50. Average Survivin expression in HCCs, and paired background livers.

There remains a greater expression of Survivin in tumours compared to paired background liver samples, when quantified using the Allred score (mean tumour 5.1 compared to background, 3.3, t(42)= 8.8, p= 3.8E-11). As has been shown above, the

Survivin quantification has, on average, been lower than that detected in other tumour types. An in-depth analysis to ascertain the role of Survivin in HCCs will show correlations with some malignant characteristics.

To further clarify the role of Survivin in HCCs and any potential relationships between adverse clinical measures, table 51 highlights significant differences between groups.

Clinical measures: HCC	Significance of Allred Score Differences Between Groups
Fibrosis	P= 0.142
Cirrhosis	P= 0.238
Stage I & II vs III & IV	P= 0.106
Vascular Invasion	**P= 0.013**
Tumour Grade*	**P= 0.007**

Table 51. Differential Survivin expression, based on clinic-pathological characteristics in HCC, when FL-HCCs are excluded.

Survivin expression differs significantly between tumours with vascular invasion (p= 0.013) and between tumour grades, p= 0.007. Figures 62 and 63 assess these differences further.

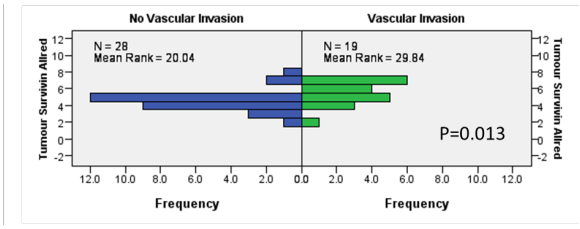


Figure 62. HCCs with vascular invasion express more Survivin.

As figure 62 demonstrates, the Allred score mean ranks for VI^+ tumours are 29.8 vs 20.1 (VI^-) U= 377, p= 0.013. Median Survivin is greater in VI^+ tumours (6.0) compared with their VI^- counterparts (5.0).

As can be seen from figure 63, there is significantly more Survivin staining in mild/moderately differentiated HCCs (mean rank 26.6, median 5.0) compared to well differentiated tumours (9.4, median 4.0) χ^2 (2) = 10.037, p= 0.005. Interestingly there is

minimal increased Survivin expression in poorly differentiated tumours (mean rank 26.3, median 5.0) compared with MMD (p= 1.00) and WD tumours, p= 0.087. These differences represent an increase in apoptosis inhibitor quantity in tumours of less favourable differentiation.

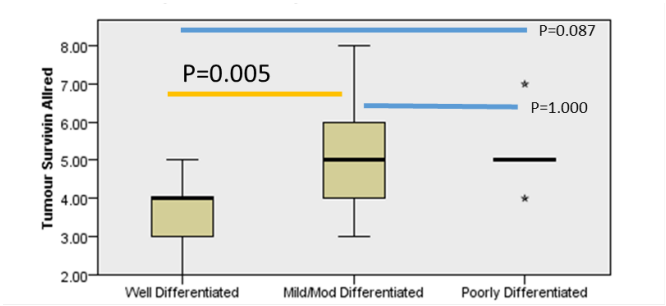
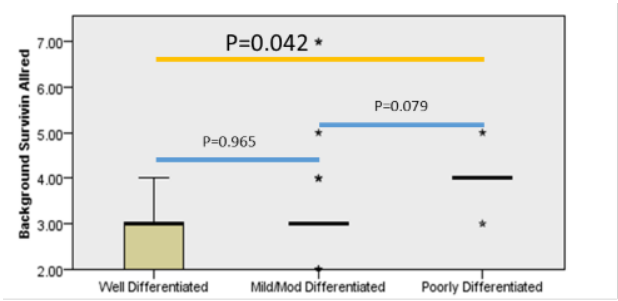


Figure 63. Survivin expression in HCCs can discern well-differentiated tumours from mild/moderately differentiated HCCs.

There was no correlation between Survivin expression and the patient's age (p= 0.085), or tumour size, p= 0.448. High expression of tumour Survivin is not associated with cumulative survival, χ^2 (1) = 2.225, p= 0.136. Median survival in Survivin^{Low} HCCs was 62.0 +/- 27.3 months (95% CI 8.4 – 115.6) did not differ significantly when compared with Survivin^{High} HCCs 24.0 +/- 15.0 (95% 0.0 – 53.4).

Survivin expression in the background liver from which various tumour grades grew from can be seen in figure 64. Survivin expressed by the background liver varied significantly depending on the tumour grade that subsequently grew, χ^2 (2) = 6.692, p= 0.035. The Survivin mean rank of background liver from which well differentiated tumours grew (14.80) was significantly lower than livers which gave poorly differentiated HCCs (30.80, p= 0.042).



Tumour Grade: **Hepatic** Survivin	**N**	**Mean Rank**	**Mean +/- S.D.**	**Median**
Well Differentiated	5	14.8	2.8 +/- 0.8	3.0
Mild/Moderately Differentiated	30	19.7	3.3 +/- 1.2	3.0
Poorly Differentiated	5	30.8	4.0 +/- 0.7	4.0

Figure 64. Levels of Survivin in **background liver** samples may be significantly different between livers that go on to develop well versus poorly differentiated tumours.

Categorising HCCs into high/low Survivin expressers based on Allred score showed a correlation with high Survivin and both tumour stage and vascular invasion – see table 52.

HCC		N	Survivin^{Low} % (n)	Survivin^{High} % (n)	P-value
Fibrosis	**-**	26	69.2 (18)	30.8 (8)	0.755
	+	26	76.9 (20)	23.1 (6)	
Cirrhosis	**-**	36	69.4 (25)	30.6 (11)	0.506
	+	16	81.3 (13)	18.7 (3)	
Tumour Stage	**I & II**	29	86.2 (25)	13.7 (4)	**0.007**
	III & IV	17	47.1 (8)	52.9 (9)	
Tumour Stage*	**I**	21	90.5 (19)	9.5 (2)	**0.026**
	II	8	75 (6)	25 (2)	
	III	14	50.0 (7)	50.0 (7)	
	IV	3	33.3 (1)	66.7 (2)	
Grade	**Well**	8	100 (8)	0.0 (0)	0.130
	Mild/ Mod	35	65.7 (23)	34.3 (12)	
	Poor	5	80 (4)	20 (1)	
Vascular Invasion	**-**	29	89.7 (26)	10.3 (3)	**0.002**
	+	19	47.4 (9)	52.6 (10)	
pSurv Allele	**CC**	4	50.0 (2)	50.0 (2)	0.172
	CG	23	87.0 (20)	13.0 (3)	
	GG	23	69.6 (16)	30.4 (7)	

Table 52. High tumour Survivin correlates with the clinico-pathological characteristics in HCCs.

High Survivin was significantly associated with vascular invasion and more advanced disease. Just over half the tumours that invade the vascular network (VI^+) express high levels of Survivin (10/19, 52.6%) compared to around 1 in 10 of less invasive tumours (3/29, 10.3%, p= 0.002). High Survivin is expressed in 52.9% (9/17) of advanced stage, compared to 13.8% (4/29) of early-stage disease, p= 0.007. When comparing individual tumour stages, more express high Survivin at a more advanced stage with 9.5% (2/21, stage I), 25% (2/8, II), 50% (7/14, III) and 66.7% (2/3) of stage IV tumours having high tumour Survivin levels, $\chi^2(3) = 9.124$, p= 0.026. This stepwise increase in Survivin expression across adversely associated tumour grades suggests a possible role for this apoptosis inhibitor in HCC progression.

4.1.5.6 A Note on FL-HCCs

There was preferential expression of Survivin by FL-HCCs (median Allred score: 7.0), differentiating them from HCCs. Given the clear difference in Survivin expression between HCCs and FL-HCCs, as well as the different clinical presentation (younger patients, not associated with cirrhosis and only a single death within the follow up period) these tumours have therefore have been excluded from the HCC analysis. When considering a small cohort of six tumours, there are no significant correlations between Survivin expression and any clinical parameter, including cumulative survival.

4.1.5.7 CCA

The Cholangiocarcinomas also express significantly more Survivin in tumour, compared to paired background liver samples as shown in table 53.

CCA Allred Score	Tumour	Background Liver
Mean +/- S.D.	5.3 +/- 1.4	3.1 +/- 0.9
Min – Max (range)	2 – 8 (5)	2 – 5 (3)
Median	6.0	3.0
Test Statistic	t(26) = 7.3, p= 9.4E-8	

Table 53. Average Survivin expression in CCAs and paired background liver tissues.

Unlike in HCC, in CCA differences in tumour Survivin protein levels were associated with the promoter variants of *rs9904341*, figure 65, p= 0.047. The mean ranks of CC (6.67), CG (17.2) and GG (22.17) are significantly different (p= 0.047) with particular difference between the homozygous alleles, as outlined in figure 65, when pairwise comparisons are made.

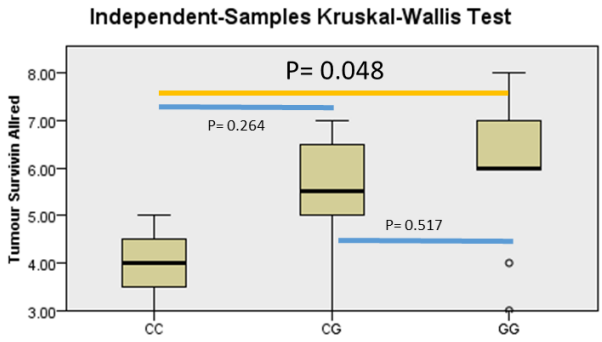


Figure 65. Survivin promoter variants differentially express Survivin protein In CCAs.

Comparisons of Survivin expression between groups (based on clinical measures) is outlined in table 54.

Clinical measures: CCA	Significance of Allred Score Differences Between Groups
Outcome	**P= 0.005**
Fibrosis	P= 0.092
Stage I & II vs III & IV	**P= 0.024**
Vascular Invasion	P= 0.310
Tumour Grade*	P= 0.291
Perineural Invasion	**P= 0.045**

Table 54. Survivin expression in all CCAs varies based on clinico-pathological characteristics.

Tumour Survivin levels appear to differ significantly in all-cause mortality (outcome), early vs late-stage disease and perineural invasion. Allred scores for Survivin expression in early and late-stage disease is shown in figure 66.

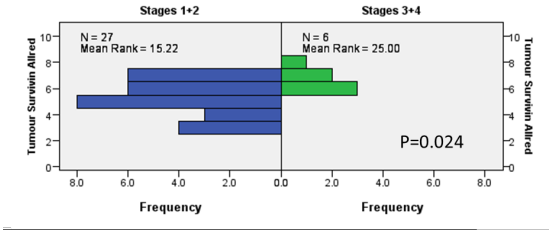


Figure 66. Higher Survivin expression in more advanced stage disease in CCA.

Tumour Survivin is lower in early-stage disease (mean rank 15.2, median 5.0) compared with more advanced disease (mean rank 25.0, median 6.0), in this study cohort, U= 129, p= 0.024.

CCA has a propensity to spread through the neural network (perineural invasion) of the liver and this feature was associated with higher levels of Survivin expression, figure 67.

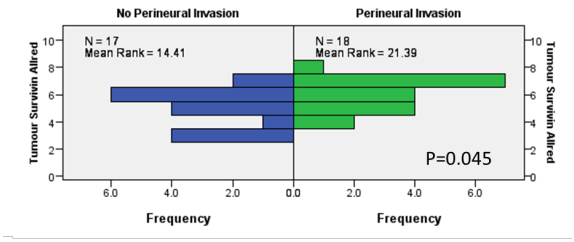


Figure 67. Higher Survivin levels may be associated with perineural invasion in CCA.

The presence or absence of perineural invasion ($PI^{+/-}$) was recorded in 35 of the CCAs included in this study. Of the 35 CCAs included, the Allred score mean rank for those with perineural invasion (21.4) was significantly higher than those tumours without perineural invasion (14.4, U= 214, p= 0.045). Whilst this is a small cohort and includes both intrahepatic and perihilar CCA, it's interesting to see this adverse clinical measure associate with higher Survivin levels and that this is also reflected in Survivin median values (PI^{+} 6.0, PI^{-} 5.0).

There is a non-significant trend towards fibrosis-related CCAs expressing more tumour Survivin when compared with their non-fibrotic counterparts using Allred-score, U= 146, p= 0.092. However, for all CCAs there was no correlation between the scale variables age (rho = 0.291, p= 0.089) and tumour size (rho = 0.181, p= 0.299).

4.1.5.8 Survival data and Survivin Expression

Quantified levels of Survivin protein and how these differ across all-cause mortality are outlined in figure 68.

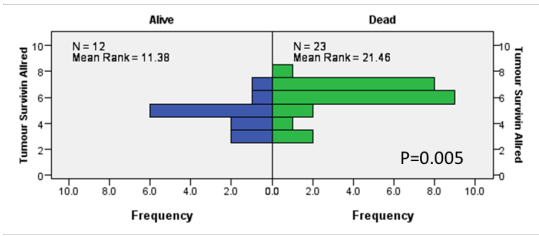


Figure 68. Higher tumour Survivin levels are associated with all-cause mortality. .

Comparing the Allred score mean ranks (alive 11.4, dead 21.5, U= 217.5, p= 0.005) demonstrates that higher Survivin level, as it was for HCC, is associated with all-cause mortality. This is supported by median CCA Survivin being lower in those patients still alive (5.0) compared to those who have subsequently died (6.0). Cumulative survival analysis with CCA categorised into high and low Survivin groups is shown in figure 69.

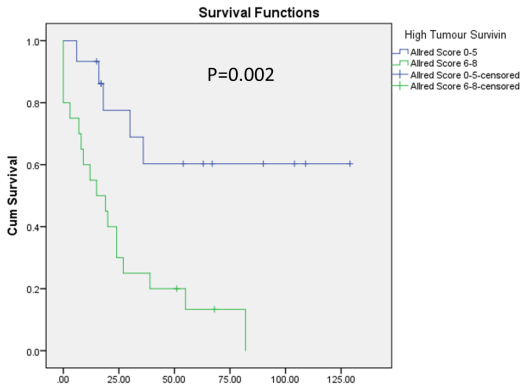


Figure 69. Cumulative survival differences based on Survivin expression in CCA.

There is a significant reduction in overall survival in CCA that express high levels of Survivin, χ^2 (1) = 9.823, p= 0.002. Median survival in Survivin^{High} CCA is 15.0 +/- 7.8 months (95% CI 0.0 – 30.3) compared to an overall median survival of 24 +/- 6.7 months (95% 10.9 – 37.1). As 60% of those with Survivin^{Low} CCAs are still alive, a median value (i.e., 50% survival) has not yet been reached, and cannot therefore be commented upon. However, differences in mean survival in Survivin^{Low} (mean 86.6 +/- 15.0 months, 95% CI 57.3 – 115.9) and Survivin^{High} (mean 25.0 +/- 6.2 months, 95% CI 12.8 – 37.1) tumours make it a potential biomarker for assisting in prognosticating survival in CCA.

4.1.5.9 Survivin^{High} and Clinicopathological Correlates in CCA

Clinical correlations and low/high expressing CCAs are shown in table 55.

CCA		N	Survivin^{Low} % (n)	Survivin^{High} % (n)	P-value
Outcome	**Alive**	12	83.3 (10)	16.7 (2)	**0.001**
	Dead	23	21.7 (5)	78.3 (18)	
Fibrosis	**-**	26	53.8 (14)	46.2 (12)	0.053
	+	8	12.5 (1)	87.5 (7)	
Tumour Stage	**I & II**	27	55.6 (15)	44.6 (12)	**0.021**
	III & IV	6	0.0 (0)	100 (6)	
Tumour Stage*	**I**	5	80.0 (4)	20.0 (1)	**0.048**
	II	22	50.0 (11)	50.0 (11)	
	III	5	0.0 (0)	100.0 (5)	
	IV	1	0.0 (0)	100.0 (1)	
Grade	**Well**	1	0.0 (0)	100.0 (1)	1.000
	Mild/ Mod	23	47.8 (11)	52.2 (12)	
	Poor	9	44.4 (4)	55.6 (5)	
Vascular Invasion	**-**	8	62.5 (5)	37.5 (3)	0.418
	+	25	40.0 (10)	60.0 (15)	
Perineural Invasion	**-**	17	52.9 (9)	47.1 (8)	0.315
	+	18	33.3 (6)	66.7 (12)	
pSurv Allele	**CC**	3	100.0 (3)	0.0 (0)	**0.021**
	CG	20	50.0 (10)	50.0 (10)	
	GG	12	16.7 (2)	83.3 (10)	

Table 55. High Survivin expression in CCAs, and clinic-pathological characteristics.

All-cause mortality is linked with high tumour Survivin, with 78.3% of those patients that went on to die expressing high levels, compared with just 16.7% of patients still alive, p= 0.001. One hundred percent of the advanced stage tumours expressed high levels of Survivin compared with 44.4% of early-stage tumours, p= 0.021. When assessing each individual CCA stage, and comparing high Survivin expression, a stage-dependent pattern emerged. Twenty percent (1/5) of stage I tumours expressed high

Survivin levels which increased with more advanced tumours, as shown by increased Survivin expression by stage II (11/22, 50%), III (5/5, 100%) and IV (1/1, 100%) tumours, $\chi^2(3) = 7.590$, p= 0.048.

Unlike in HCC an allele-dependent expression of Survivin was demonstrated in CCA. The CC allele tumours only expressed low levels of Survivin (i.e., 0% high Survivin expression) with 50% (10/20) of CG heterozygotes and 83.3% (10/12) of the GG homozygotes expressing high Survivin levels, χ^2 (2) = 7.778, p= 0.021.

4.1.5.10 Differentiating CCAs: Perihilar vs. Intrahepatic

There was no difference in Survivin expression between iCCA and pCCA tumour samples, p= 0.654. However, when comparing the background liver levels of Survivin, a significant difference becomes apparent.

Of the 36 CCAs studied 28 had background liver tissues available for assessment. Four of these were from pCCAs, which demonstrated significantly higher Survivin expression compared to the background livers from iCCAs. Quantification found a mean rank iCCA (13.21) lower than pCCA (22.25) to a significant extent, U= 79, p= 0.042. The significance of this difference is uncertain, but could possibly be related to chronic inflammation from, for example, biliary stasis that is known to occur more frequently in perihilar, and distal CCAs. Further work is required to clarify these findings.

4.1.5.11 iCCA

Analysing the iCCAs as a separate group found similar correlations as the CCA cohort overall. Mean Allred scores for tumour (5.3) compared with background liver (2.9, t(22)= 7.4, p= 2.3E-7) confirm that Survivin is expressed more in iCCA tumours compared to background liver samples, with no difference in Survivin expression based on the three *rs9904341* alleles, p= 0.143.

As in the CCA cohort, Survivin expression in iCCA was associated with tumour stage (figure 70, p= 0.026), perineural invasion (Mean ranks PI^{+}18.7, PI^{-} 12.4, U= 146.5, p= 0.048) and increased mortality (mean rank dead 18.1, alive 9.9, U= 155.5, p= 0.009).

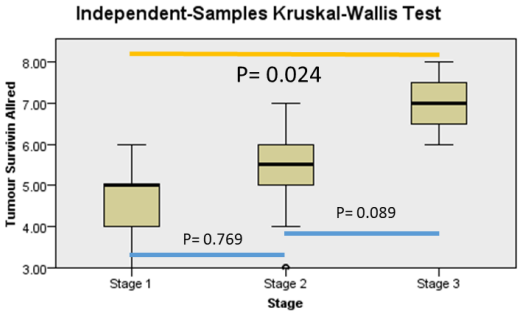


Figure 70. Survivin expression differentiates between stage I and stage III disease in iCCA.

$Survivin^{High}$ expressing iCCA was also shown to be associated with reduced cumulative survival, χ^2 (1) = 6.934, p= 0.008, figure 71.

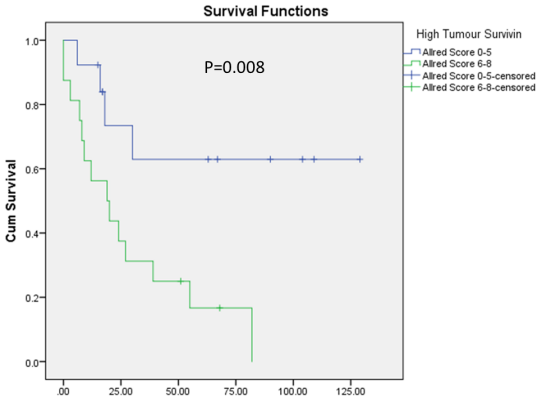


Figure 71. High Survivin expressing iCCA have a reduced cumulative survival.

Median survival in $Survivin^{High}$ iCCA is 19.0 +/- 8.0 months (95% CI 3.3 – 34.7) compared to an *overall survival* of 27.0 +/- 8.0 months (95% CI 11.3 – 42.7). Average survival for $Survivin^{Low}$ (>50% still alive therefore median statistics from KM are not calculated. However, mean Survival for $Survivin^{Low}$ is 88.0 +/- 16.6 months (95% CI 55.6 – 120.5) which is greater than that of $Survivin^{High}$ iCCAs (28.8 +/- 7.4 months, 95% CI 14.3 – 43.2).

4.1.5.12 Mixed

There were only seven mixed CCA-HCCs for comparison which are of particular clinical interest owing to their mixed cell-lineage. As there are so few of these mixed tumours this does limit the strength of any conclusions.

As for all other PLCs Survivin expression was higher in tumour than background liver table 56.

Mixed Allred Score	Tumour	Background Liver
Mean +/- S.D.	5.3 +/- 1.1	3.7 +/- 1.2
Min – Max (range)	4– 7 (3)	3– 6 (3)
Median	5.0	3.0
Test Statistic	Z = -2.2, p= 0.026	

Table 56. Average Survivin expression in mixed tumour and paired background liver.

The Allred score mean differences were also significantly higher in tumour samples (5.3) compared to background liver (3.7, Z= -2.232, p= 0.026). All of the mixed tumours were *rs9904341* CG heterozygotes so comparisons between allele's Survivin levels was not possible.

Of the 7 mixed tumours, 3 were high expressors of Survivin (Allred 6-8). In this small study subset of samples, there were no significant differences in the distribution of Survivin expression between any groups (based on clinical characteristics). Mixed tumours that expressed high levels of Survivin were not associated with a change in cumulative survival, χ^2 (1) = 0.856, p= 0.355.

All of the tumours (3/3, 100%) from patients with metabolic risk factors expressed high levels of Survivin, with the converse being true of those without metabolic risk factors (i.e., 0/4, 0%) p= 0.029.

4.1.5.13 Adenomata

The benign Adenomas also expressed more Survivin in tumours tissue than paired background liver using the Wilcoxon signed rank method, as shown in table 57.

Adenoma Allred Score	Tumour	Background Liver
Mean +/- S.D.	4.9 +/- 1.5	3.5 +/- 0.7
Min – Max (range)	3 – 7 (4)	3 – 5 (2)
Median	5.0	3.0
Test Statistic	Z = -2.3, p= 0.024	

Table 57. Average Survivin expression in Adenomas and paired background liver tissues.

Allred score median values for tumour Survivin (5.0) is greater than in background liver (3.0, Z= -2.263, p= 0.024). No correlation was seen between Survivin levels and clinical characteristics, patient age (rho= 0.204, p= 0.571), tumour size (rho= -0.168, p= 0.642) or the promoter *rs9904341*. As expected with benign tumours, all patients were alive at end of follow up, so there were no survival differences.

4.1.6 Discussion

Using a digital pathology platform to quantify expression of a single protein (Survivin) has been achieved in both tumour and paired background liver samples using whole slide images. With neoplastic expression of Survivin is consistently higher in both benign and malignant neoplasms, it appears to be a method deployed during the neoplastic growth of liver tumours, whether they are benign or malignant growths. This is analogous to previous reports of gastric carcinoma expresses more Survivin compared to normal gastric mucosa (Gu *et al.*, 2014), as well as Survivin being reported as expressed by both benign and malignant ovarian neoplasms, (He et al., 2018).

4.1.6.1 Major Findings

Digital approaches for quantification and stratification of Survivin expression have discovered correlations between clinical characteristics and protein expression at the

level of the entire cohort and in tumour subgroup analyses. When considering the whole study cohort (including the benign Adenomas), Survivin is present at higher levels in tumours of those patients with a poorer prognosis – those that die sooner, demonstrating its role in overall survival, p= 0.011.

Further to this, elevated tumour Survivin has been consistently demonstrated to correlate with clinical characteristics including vascular invasion (p= 0.008) and all-cause mortality (p= 0.016) and positively correlated with tumour size (p= 0.046) in the malignant tumours under study. However, when subsequent size (and age) correlations between Survivin expression are explored in tumour subtypes, there is no further evidence in support of these initial findings. This may be because the sample size is simply too small, or indeed it could be an example of a false discovery due to serial analyses.

Within the two main tumour sub groups (HCC and CCA) there is a divergence of clinical behaviours when it comes to Survivin-expressing tumours. CCAs express more Survivin than classical HCCs (p= 0.028), which explains the somewhat anomalous correlation of cirrhosis and *lower* tumour Survivin, p= 0.039. More HCCs (compared to CCAs) develop in the context of cirrhosis so it seems sensible that the data reflects this. It is also possible that cirrhotic liver is less able to inhibit apoptosis given then increased cellular turnover as a result of the chronic inflammatory process. Therefore, tumours from non-cirrhotic tissues are perhaps more able to react to (and overcome) physiological control mechanisms, such as apoptosis, given their inflammation-naïve state. However, this cohort is not representative of all PLCs, owing to the selection bias inherent in this surgically managed group of patients, potentially limiting the transferability of these results. It is important to remember that FL-HCCs expressed the most Survivin of all the tumours under study. As these tumours are both clinically (i.e.,

present in younger, non-cirrhotic patients) and morphologically distinct from classical HCCs, this perhaps explains the inverse relationship to Survivin expression.

When assessing HCCs, there is a strong correlation between Survivin expression and tumour grade (p= 0.007) as well as vascular invasion (p= 0.013) but no adverse survival associated with Survivin-expression, p= 0.246. Vascular invasion has recently been reported as being associated with Survivin expressing HCCs, similar to my findings (Kapiris *et al.*, 2019). More historical data has correlated Survivin expression with VI, local recurrence and disease-free survival, but not tumour grade, (Fields et al., 2004). It is clear that Survivin expression is related to some HCC tumour characteristics, but further work is required to fully ascertain these relationships.

The three grades of HCC (well, mild-moderately and poorly differentiated) express significantly different amounts of Survivin χ^2 (2) = 10.032, p= 0.007. This highlights a novel, potentially useful method of stratifying well differentiated tumours from mild/moderately differentiated has been highlighted in this study based on their ability to express Survivin. Significantly lower levels of tumour Survivin were found in well differentiated HCCs (compared to mild/moderately differentiated HCCs) using the Allred score, p= 0.005. Further to this, the level of Survivin expressed by the *background liver* varied significantly depending on the tumour grade that it subsequently generates, p= 0.035. The Survivin in background livers from which well differentiated tumours grew was significantly lower than livers which gave poorly differentiated HCCs, p= 0.042. Thus, quantifying the Survivin in background liver tissue could help identify poorly differentiated HCCs – which could be a useful aid when fully assessing the histopathological characteristics.

When considering Survivin expression in CCAs – elevated expression is associated with a poor outcome (p= 0.005), advanced tumour stage (p= 0.024), perineural invasion

(p= 0.045). As CCAs are less common than HCCs, and generally confer a poorer prognosis Survivin expression could be clinically useful to identify higher risk tumours to help monitor future surgically managed patients. There is limited published data on the role of Survivin in CCA, with a study from 2004 in a cohort of 24 patients being one of the largest other groups having been studied which also found poorer outcomes in patients with high levels of expression, (Javle *et al.*, 2004). As such, the work I have presented here contributes significantly to the body of knowledge and further outlines the clinical parameters correlate with high tumour Survivin expression.

4.1.6.2 Other Findings

Further evidence of malignant characteristics has potentially been demonstrated when considering tumour stage. Using the Allred method for quantification there appears to be more Survivin present in advanced stage (III and IV) malignancies compared with the earlier stage tumours, U= 1012, p= 0.047. An important caveat to consider here is the fact that tumour stage is being compared across tumour types, which is not typically done clinically. However, it is interesting to compare this finding with the tumour sub-group analyses. Elevated levels of Survivin have previously been associated with advanced grades of cervical cancer, renal cell cancer and HCCs (Baytekin *et al.*, 2011; Liu *et al.*, 2015; Kapiris *et al.*, 2019).

Oddly, the FL-HCCs expressed the most Survivin compared to HCCs (p= 0.028) yet carry a better prognosis. As the histological and clinical characteristics of FL-HCC are also very different from traditional HCCs these two entities were separated for the purposes of analysis. It is unclear why there is significantly more Survivin in this small cohort of tumours, so future work could involve increasing the numbers of FL-HCCs assessed to better understand the role of Survivin in these rare tumours.

When assessing cholangiocarcinomas, the first evidence of allele-dependant Survivin expression becomes apparent in our study cohort, with GG homozygotes expressing more Survivin, p= 0.047. These GG homozygotes have been demonstrated to have a higher risk of developing oral cancer in Taiwanese patients (Weng *et al.*, 2012)

and occur more commonly in malignant glial cell brain tumours, compared with benign intracranial neoplasms in a Turkish cohort (Kafadar *et al.*, 2018). These results back up the cell culture data, where GG homozygotes express more Survivin than other alleles, therefore it does make sense that (at least in this Western cohort of patients) the GG alleles correlate with increased Survivin expression. However, a complicating factor for this being patient ethnicity, as there is an established difference in allele frequencies depending on ethnicity based on a recent metanalysis, with more cancers in Survivin promoter CC homozygotes in Asians (but not Caucasians) adding further complexity to the data interpretation (Xu et al., 2014).

When all CCAs are included (iCCA and pCCA) significantly more Survivin has been detected in GG, compared to CC homozygotes, when using the Bonferroni correction for adjusted significance, p= 0.048. Similar results, albeit lacking statistical significance, are found when the iCCA are compared, p= 0.143. Once again; a larger cohort of patients would allow me to clarify whether this relationship further.

Advanced stage tumours expressed more Survivin when comparing stage III/IV disease with stage I/II CCAs, p= 0.024. Mechanistically it makes sense that the more advanced a tumour has spread, the more able it is to evade normal physiological control, such as programmed cell death. A relatively CCA-specific characteristic (perineural invasion) was found to when higher Survivin expression was detected with PI^+ tumours expressing more than PI^-, U= 214, p= 0.045. This is yet further evidence of Survivin expression potentially being a useful adjunct in assessing PLCs in routine clinical care.

However, this is a small cohort and further work would be required to fully describe the potential use of Survivin expression in CCAs.

Of the 36 CCAs studied 28 had background liver tissues available for assessment. Four of these were from pCCAs, which demonstrated significantly higher Survivin expression compared to iCCAs. Quantification found a mean rank for iCCA which was lower than that of pCCA, p= 0.042. It is interesting that Survivin is differentially expressed in the liver tissues, based on the anatomical location of the primary tumour. This may be secondary to inflammatory reaction than can occur in cholestasis, which is known to occur more frequently in CCAs that develop further down the biliary tree.

4.1.6.3 Survivin^{High} Expression

In Survivin^{High} expressing malignancies median survival was found to be significantly shorter compared to Survivin^{Low} expressing malignancies, further characterising adverse survival in tumours that strongly express Survivin protein, χ^2 (1) = 11.32, p=0.001. Similar results have also been previously reported in oesophageal cancer (Grabowski *et al.*, 2003). Therefore, elevated Survivin expression could be used to highlight patients who would benefit from closer follow up after surgery.

When the malignancies are stratified into Survivin$^{High/Low}$ groups based on their Allred score, more Survivin expression correlates with advanced tumour stage (III & IV, p= 0.029), as well as demonstrating a stepwise increase in protein as tumour stage increases, χ^2 (3) = 11.777, p= 0.007.

High Survivin expressing malignancies were grouped together and correlated with a reduced cumulative survival, p= 0.001. Other common associations across the entire malignant cohort between Survivin^{High} included increased expression in tumours with vascular invasion (p= 0.001) and in CCAs, p= 0.018.

In tumours that have invaded the vasculature, significantly more of these had Survivin^{High} levels of expression, further indicating the relevance of this relationship, p= 0.001. Vascular invasion has only recently been reported as being associated with Survivin expressing HCCs, similar to my PLC findings (Kapiris *et al.*, 2019).

The Survivin^{High} expressing HCCs did have a lower cumulative survival, as well as being more prevalent in late-stage disease (p=0.007) and in tumours that invaded the vasculature (P= 0.003), partly replicating the findings of Kapiris et al in their recent HCC research (Kapiris *et al.*, 2019). There is an incremental increase in Survivin^{High} expression through stages I-IV of HCCs, further clarifying the role of Survivin as an adverse clinical feature, $\chi^2(3) = 9.124$, p= 0.026. Using a different technique, but finding similar results to us, Montorsi and colleagues used real time PCR to demonstrate significantly more Survivin in HCC than background liver (P= 0.01) and that increased Survivin levels correlated with high tumour grade (P= 0.05) and vascular invasion (P= 0.005) (Montorsi *et al.*, 2007).

Whilst Survivin^{High} CCAs are associated with all-cause mortality (p= 0.001) and a reduced cumulative survival (p= 0.002) and a more advanced stage of disease, p= 0.021. In CCAs tumour Survivin was linked to all-cause mortality, p= 0.005. Splitting CCAs into Survivin$^{Highl/Low}$ expression has confirmed this, with Survivin^{High} having a significantly shorter overall survival, χ^2 (1) = 9.823, p= 0.002.

4.1.7 Conclusion

Quantifying Survivin expression in liver cancers has been shown to correlate with adverse clinical measures, including overall survival, in this study cohort. This early evidence suggests there may be a role in quantifying Survivin expression during the routine clinical diagnostic work up to highlight high risk patients. When considering

the main liver cancer sub-types, different characteristics correlated with elevated Survivin levels in both the tumour, and occasionally the background liver samples.

4.1.8 Further Work

It has been possible to commence liver cancer research in the Southwest region, whilst including relatively small numbers for research, whilst using novel histological quantification methods. Further work could include expanding the study cohort to include patients that are surgically resected at another hospital, or even using local tumour biopsy samples. Both of these avenues have potential logistical limitations (geographical and potentially exhausting the tissue archive respectively) but would help by further clarifying my initial findings.

Given the promising results from the Survivin expression in tumours, it would seem sensible to fully explore whether this protein is detectable in the circulation. Whether circulating tumour DNA or, as outlined below, other haematological assays could be used to quantify this. Correlations between Survivin expression in peripheral samples as well as in tumour will only be possible in a prospectively recruited cohort. Greater understanding of the biological processes involved in apoptosis inhibition would also be of merit, warranting further work in this field. Specific investigations into the role of epigenetic alterations such as promoter methylation status, and whether microRNAs alter Survivin expression seem like sensible places to start (Lyu *et al.*, 2018; Rahban *et al.*, 2019).

5 Telomerase Immunohistochemistry

5.1.1 Introduction

This chapter outlines my Telomerase IHC findings. As extra optimisation steps were required in the processing and interpretation of Telomerase IHC, and the previously reported concerns surrounding Telomerase IHC data interpretation there are understandably some concerns regarding the true accuracy of these results (il Yu et al., 2017). However, during the work here, there has been internal consistency within this research project, as well as some extensive optimisation steps that have been outlined below.

5.1.2 QuPath Assessment of Tissues: Telomerase

By way of example of the 4 categories of tissue staining, figure 72 demonstrates representative HCCs with figure 73 the CCAs. Negative (-) tissue stains do have some small background staining and blue nuclei. As the colour scheme becomes warmer from yellow (+) to orange (++) and red (+++) the intensity of staining increases accordingly.

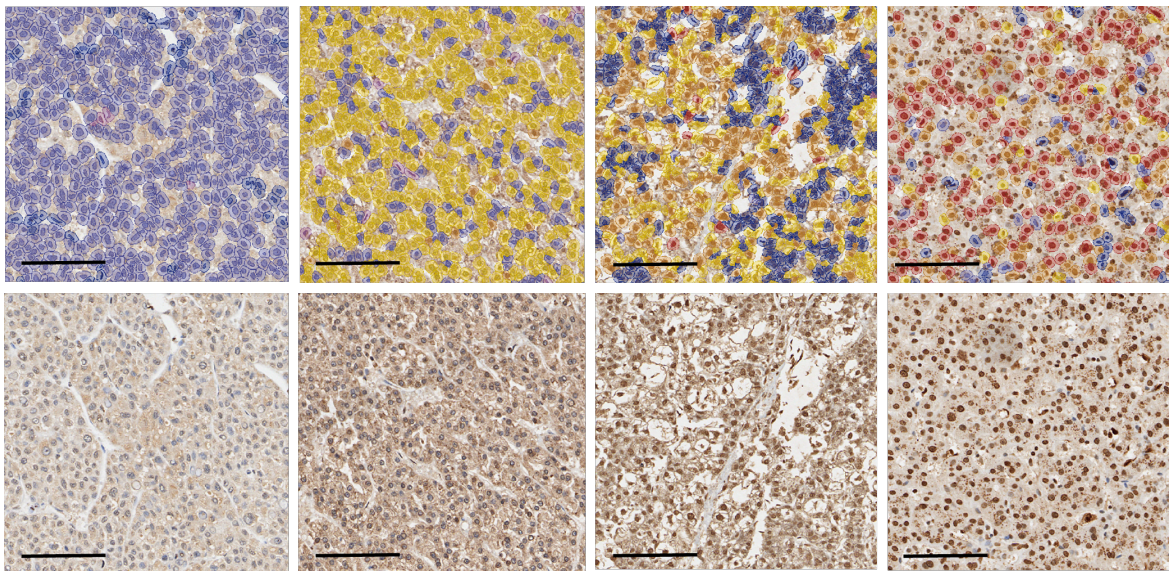


Figure 72. HCC tumours with varying degrees of nuclear positivity for Telomerase. Scale bar: 100 microns.

In figures 72 & 73 there is evidence of non-specific background staining for Telomerase that was reduced through extensive optimisation. The current QuPath software package

was only able to quantify nuclear staining intensity even though cytoplasmic expression may be present in the tissue. This is an obvious area for improvement in future work for this project.

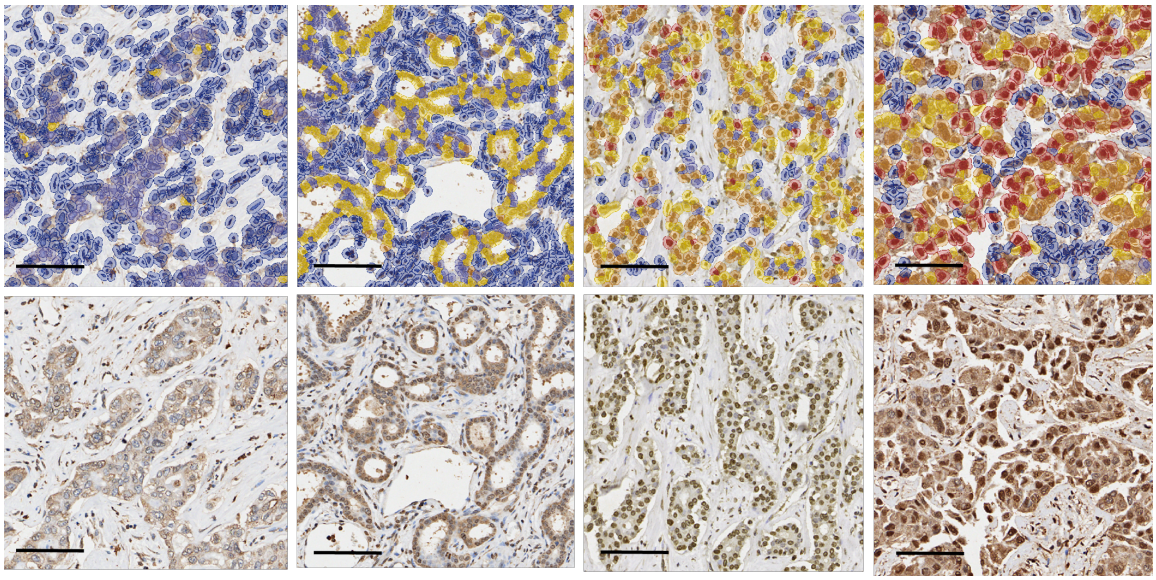


Figure 73. CCA tumours with varying degrees of nuclear positivity for Telomerase. Scale bar: 100 microns.

5.1.3 IHC Results: Telomerase

When comparing tumour versus background liver staining intensity, both the H-Score and Allred score were used initially, as shown in table 58.

All Neoplasms	**H-Score**		**Allred Score**	
	Tumour	**Background**	**Tumour**	**Background**
Mean +/- S.D.	31.6 +/- 32.5	19.4 +/- 25.7	4.1 +/- 1.1	3.6 +/- 0.1
Min - Max (Range)	0.1 – 157.2 (157.1)	0.1 – 180.2 (180.1)	2 – 7 (5)	2 – 7 (5)
Median	21.9	11.4	4.0	3.0
Test Statistic	t(94) = 3.750, p= 0.000306		t(94) = 4.272, p= 0.000046	

Table 58. Average Allred and H-Score for quantifying Telomerase expression across all neoplasms.

Data from 95 paired samples (95/111, 85.6%) assessed demonstrated a significant difference in quantified Telomerase between tumour and background liver tissues using both the H-score (t(94) = 3.750, p=0.000306) and Allred score (normally distributed, t (94) = 4.272, p=0.000046).

Assessing the role pTERT^{Mut}, and how these mutations affect the levels of Telomerase detected across all neoplasms (despite being an HCC-lineage-specific mutation) can be seen in figure 74. Comparisons between the mutational status of the tumour tissues as well as background liver tissues, some of which went on to develop a mutation were made.

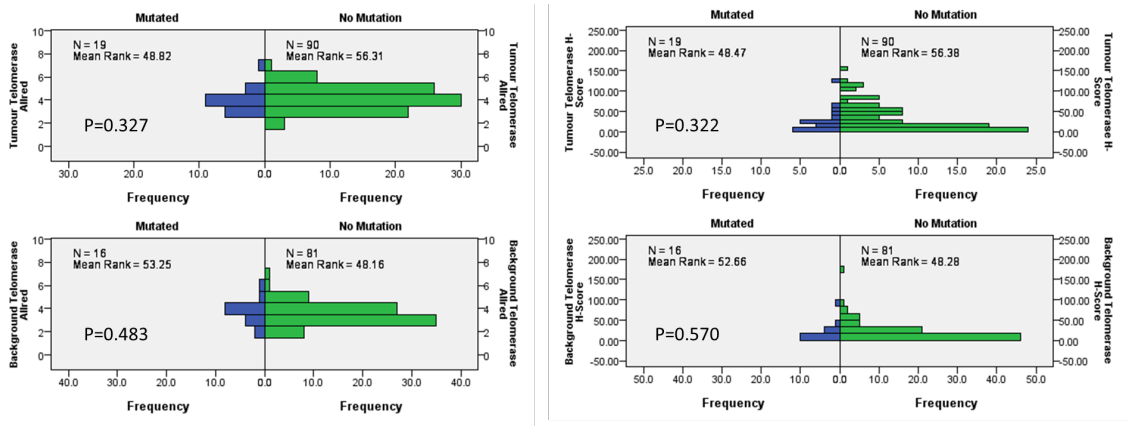


Figure 74. The effect of Telomerase promoter mutation on expressed Telomerase protein, across all neoplasms.

Across all hepatic neoplasms there is no significant relationship between Telomerase promoter mutants and detectable levels of tissue Telomerase. Furthermore, there is no difference in Telomerase levels in the background tissues near Telomerase promoter mutation containing tumours.

Differences in Telomerase levels across tumour subtypes is shown in figure 75.

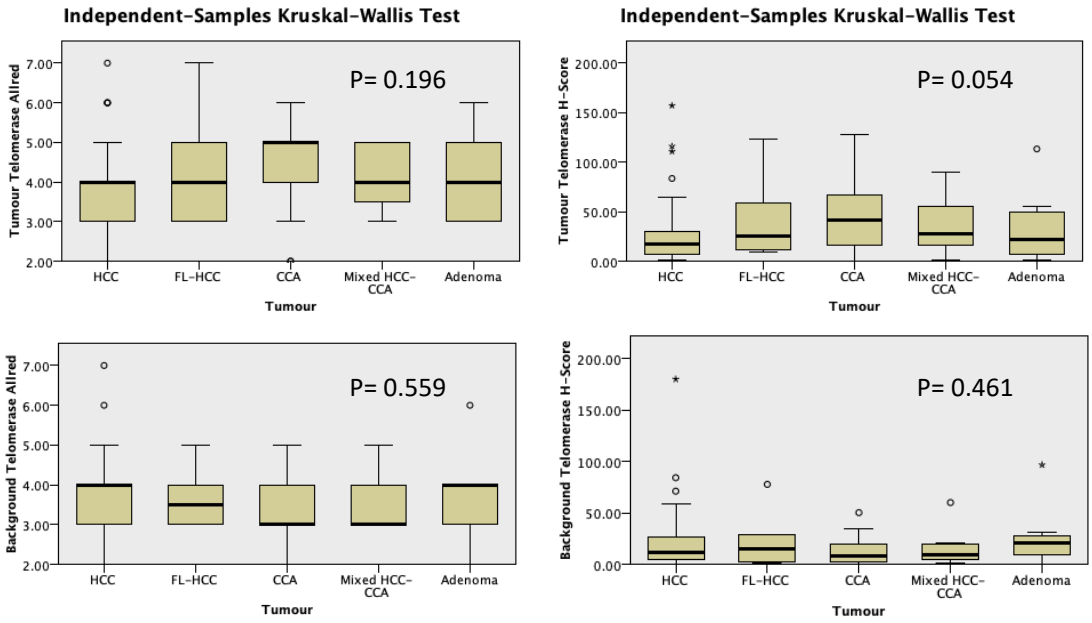


Figure 75. Tumour Survivin may be preferentially expressed in CCAs.

There were no significant differences in Telomerase expression, based on tumour subtype using either the Allred score [$\chi^2(4) = 6.048$, p= 0.196)] or the H-Score [$\chi^2(4) = 9.281$, p= 0.054].

Individual tumour scores are shown in tables 59 & 60, based on the Allred score and H-score respectively.

Allred Score	HCC	FL-HCC	CCA	Mixed HCC-CAA	Adenoma
Mean +/- S.D.	4.0 +/- 1.0	4.3 +/- 1.5	4.4 +/- 1.1	4.1 +/- 0.9	4.1 +/- 1.0
Median	4.0	4.0	5.0	4.0	4.0
Mean Rank	48.4	55.1	64.6	56.0	53.0

Table 59. Average Allred scores (0, 2 - 8) for Telomerase expression in tumour sub-types.

With CCA only expressing slightly more Telomerase than any of the other tumour subtypes, albeit to a non-significant extent, p= 0.196. These findings are replicated using the H-score below.

H-Score	HCC	FL-HCC	CCA	Mixed HCC-CAA	Adenoma
Mean +/- S.D.	26.4 +/- 32.1	42.6 +/- 44.0	44.7 +/- 32.7	37.3 +/- 31.0	33.4 +/- 34.1
Median	17.4	25.9	41.8	27.7	22.3
Mean Rank	45.7	61.3	66.2	60.3	53.6

Table 60. Average H-score values (scale 0 – 300) for Telomerase expression in tumour sub-types.

The H-score has a wider range in average values but still fails to differ between tumour subtypes, p= 0.054. The standard deviation of the Telomerase levels is consistently greater than 30.0, and sometimes greater than the mean value – indicating the results need to be interpreted with caution.

If the FL-HCC and HCCs are included in one group then the Allred score demonstrates greater Telomerase expression found higher expression is CCAs (mean rank 64.6), Mixed tumours (56.0), Adenomas (53.0) compared with HCCs (49.1), albeit to a non-significant extent, $\chi^2(3) = 5.784$, p= 0.123. However, using the H-score finds a statistically significant expression of Telomerase in tumour types, $\chi^2(3) = 7.974$, p= 0.047, with a mean rank Telomerase score of 66.2 for CCAs, 60.3 for Mixed tumours, 53.6 for Adenomas and 47.4 for HCCs. This is further assessed in figure 76.

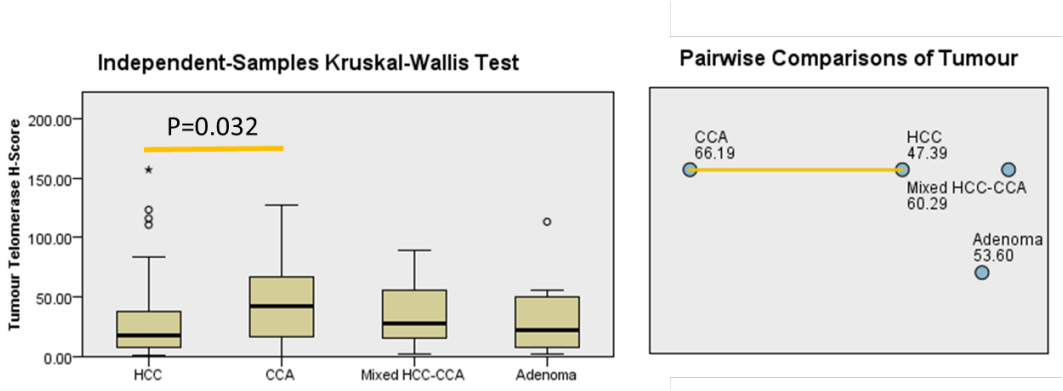


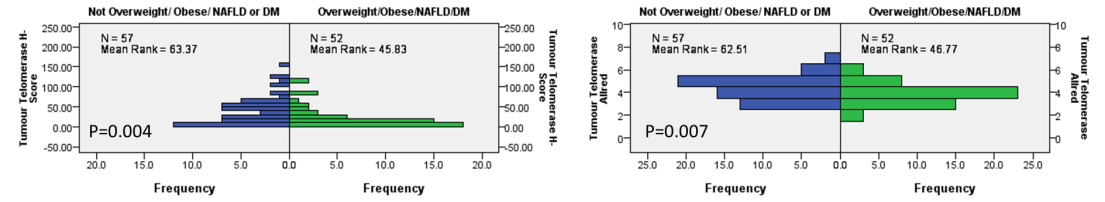
Figure 76. Tumour-specific Telomerase expression.

With mean ranks of 66.2 (CCA) and 47.4 (all HCCs) there is significantly more Telomerase in the cholangiocellular tumours, when assessing pairwise comparisons p=0.032. There is no significant difference between other tumour types, but it is

interesting to note that the mean ranks of mixed tumours (60.3) lies between that of CCAs and HCCs, albeit to a non-significant extent in this small study cohort.

The distributions of clinical measures and whether these vary with levels of Telomerase, and their respective significance was explored across all clinical parameters. The only example of any difference in the distribution of Telomerase Allred scores in these groups was when Metabolic risk factors were concerned and was replicated in both the Allred (p= 0.007) and H-score (p= 0.004).

There is a significant difference in the distribution of tumour telomerase levels in patients with, and without, metabolic risk factors. This is further assessed in figure 77.



Metabolic Risk Factors		H-Score			Allred		
Group	**N**	**Mean Rank**	**Mean +/- S.D.**	**Median**	**Mean Rank**	**Mean +/- S.D.**	**Median**
Absent	57	63.37	42.99 +/- 35.84	38.59	62.51	4.42 +/- 1.05	4.00
Present	52	45.83	25.56 +/- 28.35	16.53	46.77	3.87 +/- 0.95	4.00
Mann-Whitney U		1005			1054		
P-Value		**0.004**			**0.007**		

Figure 77. Telomerase expression in tumours is not associated with metabolic risk factors.

It appears that, across all neoplasms, there is more Telomerase detected in tumours that arise in the absence of metabolic risk factors or associated conditions when compared to patients who have metabolic risk factors (overweight, obese, non-alcoholic fatty liver disease or diabetes mellitus). Those tumours that develop in the absence of metabolic risk factors (mean ranks H-score 66.37, Allred 62.51) have significantly more Telomerase than tumours from metabolic patients (mean rank H-score 45.83, Allred 46.77) to a relatively high level of significance (H-score p= 0.004, Allred p= 0.007).

Relationship between age (years) and tumour size (mm) and the Allred or H scores is shown in table 61.

All Neoplasms		Tumour Telomerase		Background Telomerase	
		H Score	Allred	H Score	Allred
Age	Correlation Coefficient	-0.059	-0.017	-0.078	-0.070
	P Value	0.544	0.863	0.449	0.495
Tumour Size	Correlation Coefficient	-0.031	-0.028	-0.028	-0.012
	P Value	0.745	0.773	0.807	0.909

Table 61. There is no correlation between tumour/background Telomerase levels across all neoplasms and the patient age (years) or the tumour size (mm).

No significant correlations between tumour/background liver staining intensity and patient age/ tumour size were found. All correlation coefficients were negative, as shown in table 61, but lacked statistical significance.

When stratifying high and low levels of tumour Telomerase, and whether this affects cumulative survival, Kaplan-Meier curves were created to assess these subgroups, figure 78.

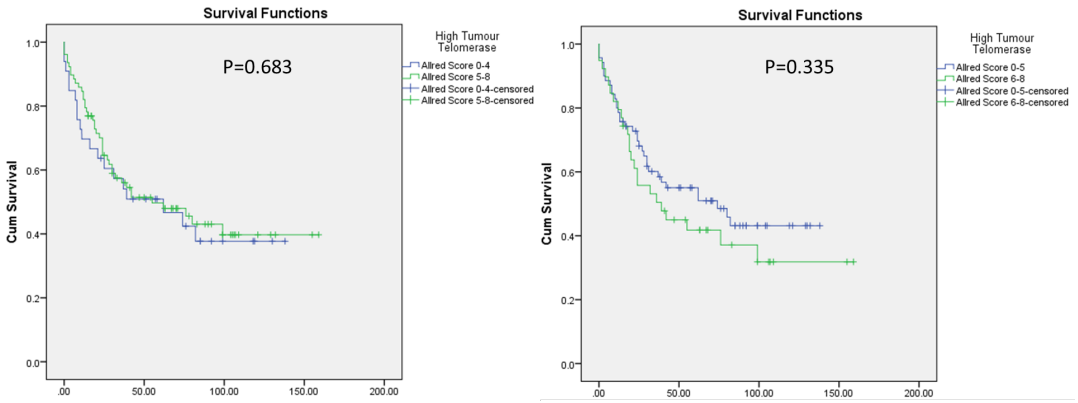


Figure 78. Telomerase expression is not associated with cumulative survival.

Both thresholds used for assessing high/low tumour Telomerase did not find a significant difference in cumulative survival. With the higher threshold for designating 'high expression' of Telomerase, there is a non-significant difference in survival, χ^2 (1) = 0.931, p= 0.335. Telomerase^{Low} expression gave a median survival of 74.0 +/- 21.8 months (31.2 – 116.8) compared to a median of 39.0 +/- 13.5 months (95% CI 12.5 – 65.5) in Telomerase^{High} expressing tumours.

When considering the more stringent high telomerase category (Allred scores 6-8), which has been used in the literature and in the Survivin analyses above, disease characteristics compared across these groups can be seen in table 62.

All Neoplasms: Telomerase		**N**	**TERT^{Low} % (n)**	**TERT^{High} % (n)**	**P-Value**
Gender	Female	44	56.8 (25)	43.2 (19)	0.224
	Male	65	69.2 (45)	30.8 (20)	
Outcome	Alive	50	70.0 (35)	30.0 (15)	0.317
	Dead	59	59.3 (35)	40.7 (24)	
Fibrosis	-	65	58.5 (38)	41.5 (27)	0.160
	+	43	72.1 (31)	27.9 (12)	
Cirrhosis	-	85	60.0 (51)	40.0 (34)	0.143
	+	23	78.3 (18)	21.7 (5)	
Viral Hepatitis	-	94	62.8 (59)	37.2 (35)	0.566
	+	15	73.3 (11)	26.7 (4)	
Metabolic Risk Factors	-	57	50.9 (29)	49.1 (28)	**0.003**
	+	52	78.8 (41)	21.2 (11)	
Genetic Liver Disease	-	99	63.6 (63)	36.4 (36)	1.000
	+	10	70.0 (7)	30.0 (3)	
pTERT Mutant	WT	90	61.1 (55)	38.9 (35)	0.190
	Mut	19	78.9 (15)	21.1 (4)	
Tumour	HCC	50	78.0 (39)	22.0 (11)	**0.027**
	FL-HCC	6	66.7 (4)	33.3 (2)	
	CCA	36	44.4 (16)	55.6 (20)	
	Mixed	7	57.1 (4)	42.9 (3)	
	Adenoma	10	70.0 (7)	30.0 (3)	

Table 62. Clinico-pathological correlates based on high Telomerase expression.

Telomerase^{High} levels are seen in 49.1% (28/57) of tumours from patients without metabolic disease such as diabetes, obesity and fatty liver disease compared with 21.2% (11/52) of those with metabolic conditions, p= 0.003. Also, Telomerase^{High} levels are also noted to occur in 55.6% (20/36) of CCA tumours compared with 42.9% (3/7) of

mixed tumours, 30.0% (3/10) of Adenomas, 22.0% (11/50) of HCCs, and in 33.3% (2/6) of FL-HCCs, χ^2 (4) = 10.573, p= 0.027.

Otherwise, there was no increased Telomerase expression in either gender, in those that have subsequently died (outcome variable), fibrosis, cirrhosis, genetic or viral hepatitis driven tumours. Malignant characteristics will be assessed below.

5.1.4 Tumour Subgroup analysis

Owing to the heterogeneous nature of the tumours studied, with clear differences between staining preference between tumour types, tumour groups, and subgroups were further analysed. For the sake of simplicity, consistency, and to avoid repetition, the Allred score has been used preferentially in subsequent analyses.

5.1.4.1 All Malignancies.

As a hallmark of cancer, and to reduce any potentially confusing bias in the analysis Telomerase levels in malignant tumours have been compared to background tissues, as shown in table 63.

All Malignancies	**Allred Score**	
	Tumour	**Background**
Mean +/- S.D.	4.1 +/- 1.1	3.5 +/- 1.0
Min - Max (Range)	2 – 7 (5)	2 – 7 (5)
Median	4.0	3.0
Test Statistic	t(84) = 4.272, p= 0.000052	

Table 63. Average Telomerase expression on malignant tumours and paired background livers.

There are increased levels of Telomerase expressed in tumour tissues than corresponding background liver sample. The Allred score mean values for tumour Telomerase (4.1 +/- 1.1) was significantly higher that background liver (3.5 +/- 1.0), t(84) = 4.264, p= 0.000052.

Between the malignancies, average Telomerase expression can be seen in figure 79.

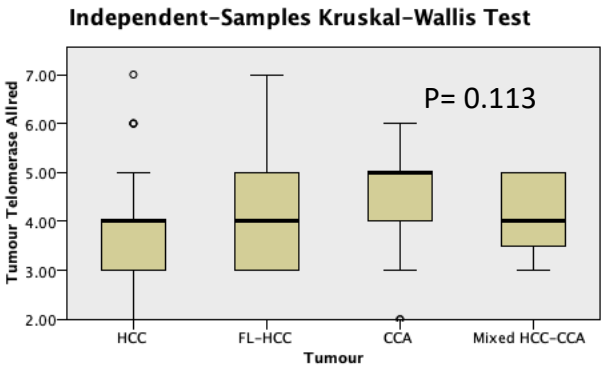


Figure 79. Telomerase expression is not tumour specific.

Of the malignant tumours, CCAs expressed the most Telomerase (mean rank 58.5, median 5.0) compared to HCCs (mean rank 43.8, median 4.0), FL-HCCs (mean rank 49.9, median 4.0) and Mixed tumours (mean rank 50.7, median 4.0) but this was not statistically significant, χ^2 (3) = 5.978, p= 0.113.

Telomerase expression and potential correlations with age and tumour size are shown in table 64. There are no statistically significant correlations between tumour size, or patient age, and the quantification of Telomerase tumour tissues.

Allred Score: All Malignancies	**Correlation Coefficient (rho)**	**P-Value**
Patient Age	-0.051	0.619
Tumour Size	0.019	0.855

Table 64. There is no correlation between tumour/background Telomerase levels and patient age, or tumour size in malignant tumours.

When exploring whether clinical characteristics are associated with differential Telomerase expression, the results can be seen in table 65.

All Malignancies	**Tumour Allred Score Distribution**
Gender	P= 0.073
Outcome	P= 0.809
Metabolic Risk Factors	**P= 0.002**

Table 65. Differential expression of Telomerase based on clinico-pathological findings.

Differences in the tumour Telomerase distribution are seen when comparing patients with, and without, metabolic disease risk factors or associated conditions (p= 0.002) and

a non-significant difference of Telomerase expression based on gender is also apparent, p= 0.073. These differences are further assessed below in figures 80 & 81.

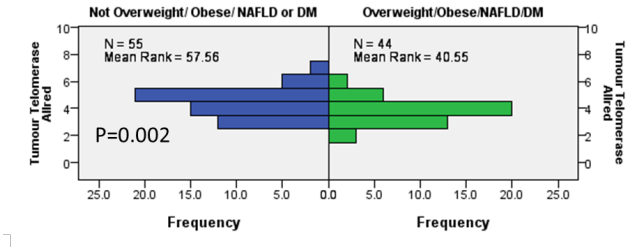


Figure 80. Higher tumour Telomerase levels are found in tumours from patients that do not have metabolic risk factors.

The Allred score demonstrates a statistically significant difference in Telomerase detected, depending on whether the patient has a metabolic condition, such as diabetes or fatty liver disease. The Allred score also finds more Telomerase in non-metabolically-derived tumours (mean rank 57.6) compared with metabolically-derived tumours (mean rank 40.6), U= 794, p= 0.002. Implying that tumours arising in patients with metabolic risk factors/associated conditions do not preferentially deploy Telomerase as a method of tumour cell immortalisation.

Comparing the effect gender has on tumour Telomerase is set out in figure 81.

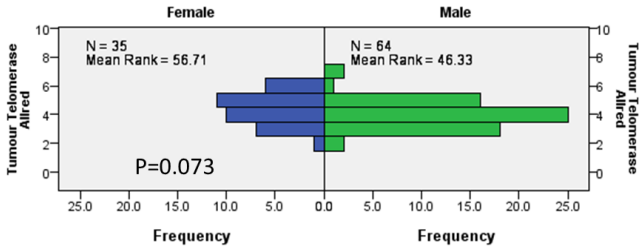


Figure 81. Higher tumour Telomerase levels may be found in malignant tumours from female patients.

When comparing mean ranks of Telomerase across all malignant tumours, there is preponderance for female patients to preferentially express Telomerase. This data, albeit non-significant, indicates that gender may play a role in Telomerase expression across liver cancers, (mean ranks: F 56.7, M 45.3, U= 885, p= 0.073).

5.1.4.2 Survival and Telomerase expression

Table 62 has already demonstrated that Telomerase quantification is not a useful tool in highlighting patients who go on to die, p= 0.809. When assessing any relationship between high tumour Telomerase and cumulative survival, Kaplan Meier survival curves can be seen in figure 82. There was no significant difference in cumulative survival observed when comparing high and low Telomerase malignancies.

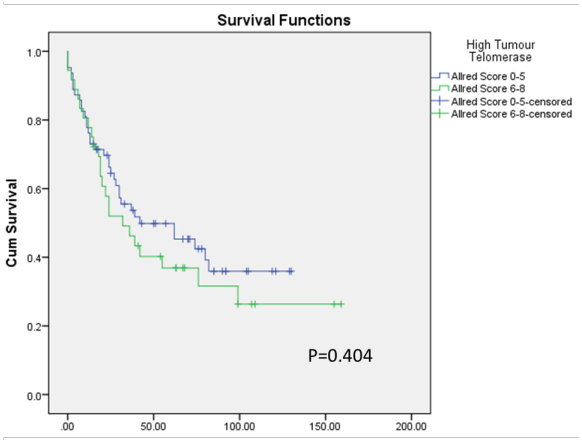


Figure 82. Telomerase levels in malignancies does not correlate with cumulative survival.

Using the more stringent cut off for designating Telomerase^{High} expression (Allred 6-8), there is a non-significant effect on cumulative survival, χ^2 (1) = 0.695, p= 0.404. Median survival 32.0 +/- 9.9 months (95% CI 12.5 – 51.5) in Telomerase^{High} tumours compared to a median survival of 42.0 +/- 21.6 (95% CI 0.0 – 84.4) in Telomerase^{Low} expressing tumours.

Correlating high tumour Telomerase and clinical characteristics highlighted two significant relationships as shown in table 66. There was a non-significant difference in high Telomerase expressing tumours based on patient gender with 48.6% (17/35) of women and 29.7% (19/64) of men expressing high levels of Telomerase protein, p= 0.081.

All Malignancies: Telomerase		N	TERT^{Low} % (n)	TERT^{High} % (n)	P-Value
Gender	Female	35	51.4 (18)	48.6 (17)	0.081
	Male	64	69.2 (45)	30.8 (20)	
Outcome	Alive	40	70.0 (28)	30.0 (12)	0.297
	Dead	59	59.3 (35)	40.7 (24)	
Metabolic Risk Factors	-	55	49.1 (27)	50.9 (28)	**0.001**
	+	44	81.8 (36	18.2 (8)	
pTERT Mutant	WT	80	60.0 (48)	40.0 (32)	0.184
	Mut	19	78.9 (15)	21.1 (4)	
Tumour	HCC	50	78.0 (39)	22.0 (11)	**0.011**
	FL-HCC	6	66.7 (4)	33.3 (2)	
	CCA	36	44.4 (16)	55.6 (20)	
	Mixed	7	57.1 (4)	42.9 (3)	

Table 66. High tumour Telomerase and clinic-pathological correlates.

Telomerase^{High} levels are found in 50.9% (28/55) of the non-metabolically driven malignancies compared to those 18.2% (8/44) from metabolic patients, p= 0.001. When comparing malignant tumour sub-types, Telomerase^{High} was found in 55.6% (20/36) of CCA compared with 42.9% (3/7) of Mixed tumours and 23.2% (11/50) of HCCs and 33.3% (2/6) FL-HCCs, χ^2 (2) = 10.411, p= 0.011.

5.1.4.3 HCCs

Fibrolamellar tumours were excluded from analysis of HCCs. Comparisons of the 44 paired tumour-background tissues are shown in table 67.

HCC Allred Score	Tumour	Background Liver
Mean +/- S.D.	3.9 +/- 1.0	3.6 +/- 1.1
Min – Max (range)	2 – 7 (5)	2 – 7 (5)
Median	4.0	4.0
Test Statistic	t(43) = 1.699, p= 0.096	

Table 67. Average Telomerase levels in HCCs and paired background liver tissues.

In the HCC only cohort there remains no significant difference between tumour and background telomerase levels. The Allred score for HCC tumour (3.9 +/- 1.0) was marginally greater than that found in background liver (3.6 +/- 1.1) meaning there is a non-significant difference between Telomerase protein in liver and tumour samples tested, t(43) = 1.699, p= 0.096. This is the first evidence of a universal tumour antigen not being differentially expressed by tumour and background liver, perhaps indicating it is not a useful tool for assessing HCCs. If the FL-HCCs and classical HCCs are included together in a single cohort there remains a non-significant difference between tumour and background liver Telomerase-expression, further emphasising this finding, t(50) = 1.997, p= 0.051.

The pTERT^{Muts} status and Telomerase protein levels in tumour and background tissues were non-significantly different, as shown in figure 83.

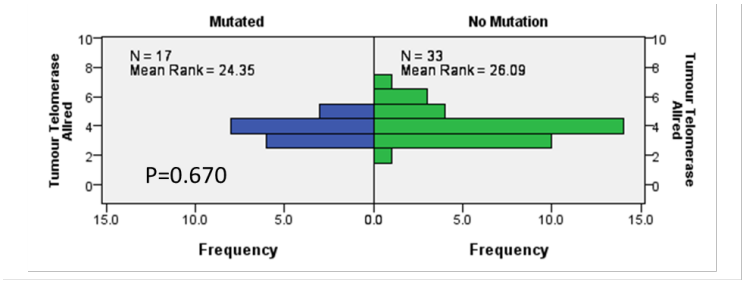


Figure 83. Telomerase promoter mutations do not result in increased Telomerase protein expression in HCCs.

HCCs containing Telomerase promoter mutants expressed less Telomerase protein (mean rank 24.35) compared to WT HCCs (mean rank 26.09) when comparing Allred scores, but this difference was not statistically significant, U= 261, p= 0.670.

There was no significant distribution in the Telomerase expression between any clinical parameter. Furthermore, there was no significant correlations between Telomerase protein and patient age (rho= 0.082, p= 0.572) or tumour size (rho= 0.062, p= 0.669).

No difference in cumulative survival for high and low Telomerase expressing HCC tumours was found, as shown in figure 84.

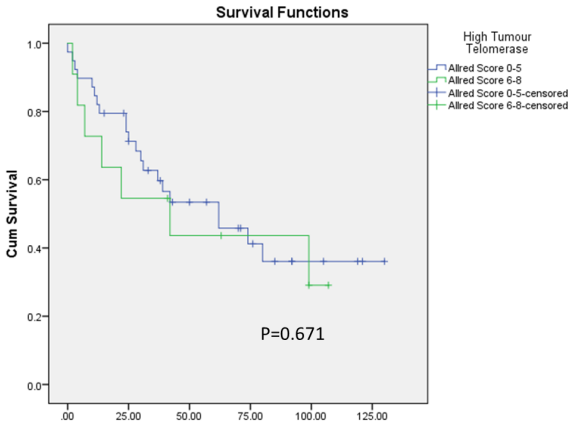


Figure 84. HCCs expression of Telomerase does not correlate with cumulative survival.

Stratifying Telomerase$^{High/Low}$ expression did not demonstrate a difference in cumulative survival, χ^2 (1) = 0.180, p= 0.671. Median of 42.0 +/- 21.7 (95% CI 0.0 – 84.5) in tumours with high nuclear Telomerase compared to a median 62.0 +/- 19.6 (95% CI 22.9 – 101.1) in low-expressing tumours did not reach statistical significance. In addition to this, there were no clinical correlations differentially expressed between Telomerase$^{High/Low}$ expressing tumour.

5.1.4.4 All CCA

There is more Telomerase in CCA tumours when comparing to paired background liver samples, as shown in table 68.

CCA Allred Score	Tumour	Background Liver
Mean +/- S.D.	4.2 +/- 1.1	3.3 +/- 0.8
Min – Max (range)	2 – 6 (4)	2 – 5 (3)
Median	5.0	3.0
Test Statistic	t(27) = 4.076, p= 0.000362	

Table 68. Average Telomerase in CCA and paired background liver tissues.

Average Telomerase protein expression was greater in CCA tumours (4.18 +/- 1.06) compared to paired background liver tissues (3.32 +/- 0.77), t(27) = 4.076, p< 0.0004.

When assessing the distribution of telomerase staining across clinically distinct groups, there are a few significant differences that have been highlighted in table 69.

Clinical measures: CCA	Significance of Allred Score Differences Between Groups
Metabolic Risk Factors	**P= 0.001**
Perineural Invasion	P= 0.481
iCCA vs pCCA	**P= 0.016**

Table 69. Telomerase expression is differentially expressed based on clinico-pathological properties.

Telomerase appears to be differentially expressed when patients have metabolic diseases, and is also significantly different between pCCA and iCCA.

The metabolically related tumours and the levels of Telomerase are shown in figure 85.

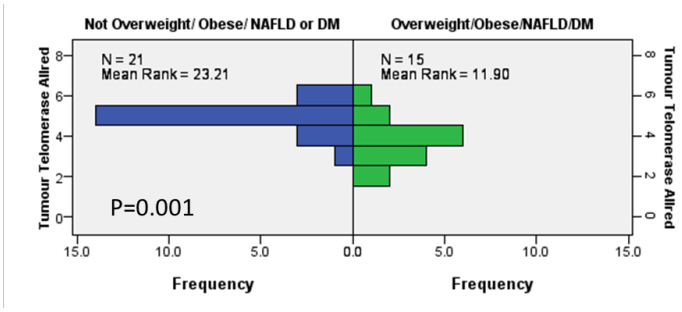


Figure 85. Higher tumour Telomerase levels are found in CCAs in patients that do not have metabolic risk factors.

Higher telomerase is found in the non-metabolically derived tumours when quantified with the Allred score (mean ranks 23.21 vs 11.90, U= 58.5, p= 0.001) registering these highly significant results.

The difference in Telomerase between perihilar (pCCA) and intrahepatic CCA (iCCA) is shown in figure 86.

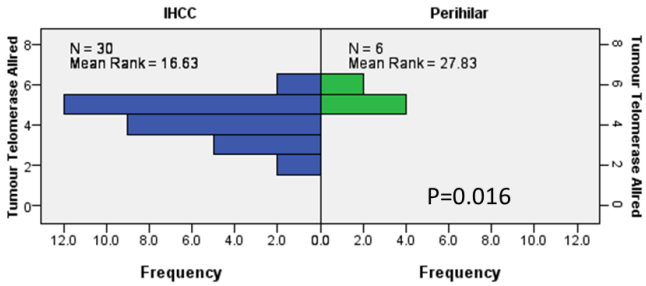


Figure 86. Higher tumour Telomerase levels are associated perihilar CCA compared to intrahepatic CCA.

Allred score (mean ranks iCCA 16.63, pCCA 27.83) demonstrates higher levels of tumour Telomerase expressed by pCCAs, based on this small study cohort, U= 146, p= 0.016.

When correlating Telomerase expression with age and tumour size, a couple of interesting results are highlighted. The levels of Telomerase in the background liver are negatively correlated with patient age. The negative correlation coefficients (Allred score rho= -0.417, p= 0.027) indicate that younger patients with CCA express more Telomerase compared to their older counterparts. No correlations between tumour Telomerase and the clinical characteristics (including tumour size) were found.

There is no significant difference between Telomerase$^{High/Low}$ expression and cumulative survival, as outlined in figure 87.

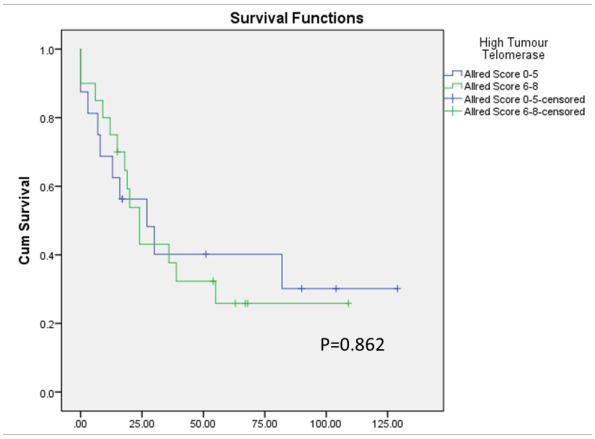


Figure 87. CCA Telomerase expression is not correlated with cumulative survival.

There is no difference in survival between high and low Telomerase expressing tumours, χ^2 (1) = 0.030, p= 0.862. Average survival for Telomerase^{High} expressing CCA tumours with a median survival of 24.0 +/- 3.5 months (95% CI 17.1 – 30.9) compared with low expressing tumours that have median survival of 27.0 +/- 11.3 (95% CI 4.8 – 49.2).

Tumours that express high levels of Telomerase are shown in table 70, with significant differences in clinical measures highlighted.

All CCA: Telomerase		**N**	**TERT^{Low} % (n)**	**TERT^{High} % (n)**	**P-Value**
Gender	Female	20	45.0 (9)	55.0 (11)	1.000
	Male	16	43.8 (7)	56.3 (9)	
Outcome	Alive	12	50.0 (6)	50.0 (6)	0.729
	Dead	24	41.7 (10	58.3 (14)	
Metabolic Risk Factors	-	21	19.0 (4)	81.0 (17)	**0.001**
	+	15	80.0 (12)	20.0 (3)	
Perineural Invasion	-	18	50.0 (9)	50.0 (9)	0.738
	+	18	38.9 (16)	61.1 (20)	
CCA Location	iCCA	30	53.3 (16)	46.7 (14)	**0.024**
	pCCA	6	0.0 (0)	100.0 (6)	

Table 70. High CCA Telomerase and clinico-pathological properties.

More of the non-metabolically related tumours (81.0%) have Telomerase^{High} compared to just 20% of metabolically related tumours, p= 0.001. Interestingly tumour location and Telomerase^{High} are related with 100% of the pCCA expressing increased levels of telomerase compared to 46.7% of iCCA, p= 0.024.

5.1.4.5 iCCA

Differentiating between CCAs based on anatomical location will help to highlight potential differences in their telomerase expression profiles. Table 71 shows that iCCA

continue to express significantly more telomerase in tumour, compared to paired liver samples.

iCCA Allred Score	Tumour	Background Liver
Mean +/- S.D.	4.0 +/- 1.0	3.3 +/- 0.8
Min – Max (range)	2 – 6 (4)	2 – 5 (3)
Median	4.0	3.0
Test Statistic	t(23) = 3.093, p= 0.005	

Table 71. Average Telomerase expression in iCCA and paired background liver tissues.

Intrahepatic CCAs express more Telomerase in tumour (4.0 +/- 1.0) than background liver (3.3 +/- 0.8) of paired samples are compared, t (23) = 3.3093, p= 0.005. The role metabolic risk factors play in telomerase expression is further explored in figure 88.

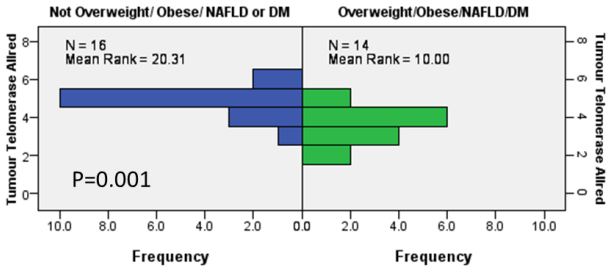


Figure 88. Higher iCCA Telomerase levels are associated with patients that do not have metabolic risk factors.

Allred scores (mean rank – 20.31, + 10.00, U = 35, p= 0.001) indicate that non-metabolically derived iCCAs express more Telomerase compared to their metabolically driven counterparts. No significant correlation coefficients were found when compared Telomerase protein levels with patient age (rho= -0.302, p= 0.104) or tumour size (rho= 0.116, p= 0.543).

Stratifying iCCA into Telomerase$^{High/Low}$ expression demonstrated no difference on cumulative survival, as shown in figure 89, χ^2 (1) = 0.050, p= 0.823.

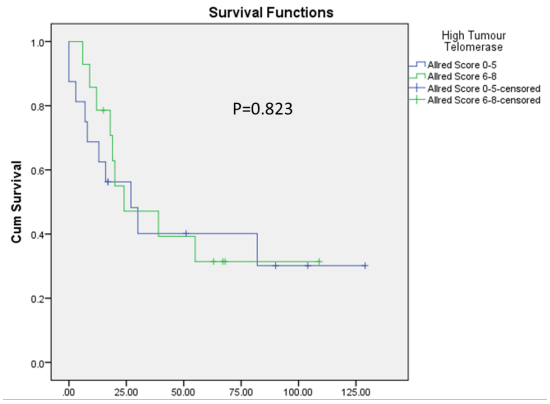


Figure 89. High Telomerase expression does not correlate with cumulative survival in iCCA.

Median survival for Telomerase^{High} is 24.0 +/- 16.7 months (95% CI 0.0 – 56.8) compared to a non-significantly increased survival in Telomerase^{Low} tumours: median survival 27.0 +/- 11.3 months (95% CI 4.8 – 49.2).

As shown in table 70 above, non-metabolically driven iCCAs expressed more Telomerase compared to metabolically driven tumours. With 75.0 % (12/16) of the non-metabolic iCCA expressing Telomerase^{High} levels compared to 14.3% (2/14) of the metabolically-associated iCCAs, p= 0.001. No other significant relationships between Telomerase$^{High/Low}$ expression and clinical characteristics was found.

5.1.4.6 Mixed

The small number of mixed tumours included in the cohort were assessed for their Telomerase-related characteristics. There was no significant difference in Telomerase expression between tumour and background liver, as shown in table 72.

Mixed Allred Score	Tumour	Background Liver
Mean +/- S.D.	4.1 +/- 0.9	3.6 +/- 0.8
Min – Max (range)	3 – 5 (2)	3 – 5 (2)
Median	4.0	3.0
Test Statistic	Z= -1.633, p= 0.102	

Table 72 Average mixed tumour and paired background liver Telomerase expression.

There is no significant difference in the distribution of telomerase protein across various clinical parameters, including pTERT^{Mut} status, p= 0.857. When considering if

Telomerase correlates with age and tumour size, there may be a positive correlation between older patients with mixed tumours, as shown in table 73.

Allred Score: Mixed Tumours	**Correlation Coefficient (rho)**	**P-Value**
Patient Age	0.794	**0.033**
Tumour Size	-0.019	0.968

Table 73. Patient age and Telomerase expression positively correlate in Mixed tumours.

The Allred score correlates positively with patient age (coefficient 0.794, p= 0.033). It is hard to interpret this data in such a small cohort size, so further work is warranted, but it appears to show that there may be a significant correlation between patient age and Telomerase expression in mixed HCC-CCA tumours.

High expression of Telomerase may be protective in the mixed tumour cohort, as shown in the cumulative survival Kaplan Meier survival plots in figure 90.

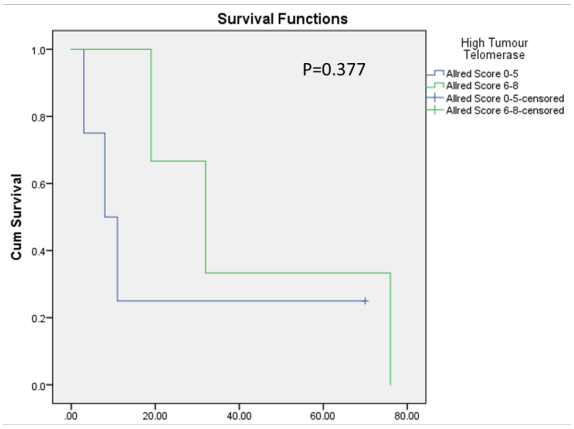


Figure 90. High levels of Telomerase in mixed tumours does not correlate with cumulative survival.

Average survival for these low expressing tumours includes a median survival of 8.0 +/- 4.0 months (95% CI 0.2 – 15.8) compared to a median survival 32.0 +/- 10.6 (95% CI 11.2 – 52.8) for the Telomerase^{High} tumours, χ^2 (1) = 0.781, p= 0.377.

5.1.4.7 Adenomas

The benign neoplasms are assessed for completeness, with irrelevant malignant characteristics excluded from analysis. There is no difference between tumour and background Telomerase, as shown in table 74.

Adenomas Allred Score	Tumour	Background Liver
Mean +/- S.D.	4.1 +/- 1.0	3.8 +/- 1.0
Min – Max (range)	3 – 6 (3)	2 – 6 (4)
Median	4.0	4.0
Test Statistic	Z= -0.749, p= 0.454	

Table 74. Average Adenoma and paired background liver Telomerase expression.

On average tumours expressed a non-significantly higher quantity of Telomerase protein compared to their paired liver samples. The Allred score gave a higher value for tumour (4.1 +/- 1.0) compared to liver tissue (3.8 +/- 1.0) but this also failed to reach statistical significance, Z= -0.749, p= 0.454. Correlations between Telomerase protein and age/tumour size are shown in table 75.

Allred Score: Adenomas	**Correlation Coefficient (rho)**	**P-Value**
Patient Age	0.750	**0.012**
Tumour Size	-0.489	0.151

Table 75. Patient age and Telomerase expression positively correlate in Adenomas.

A positive correlation exists between the age of the patient and Telomerase expression, correlation coefficient = 0.750, p= 0.012. There was no significant difference in the distribution of high Telomerase-expressing tumours between relevant clinical or pathological characteristics.

5.1.5 Discussion

The findings from my Telomerase protein detection, and correlations with clinical parameters, has not reflected previously published data. This is potentially due to technical issues surrounding the IHC protocol, or data interpretation processing on QuPath. However, it is also plausible that these findings are an accurate representation of Telomerase expression in liver tumours, but proving this would require further conformational work.

5.1.5.1 Major Findings

When assessing all neoplasms, Telomerase protein is more prevalent in tumour, compared to background liver, when using both the H-score (p= 0.000046) and Allred score (p= 0.000306) similar to previously published results (Zhou, Lu and Zhu, 2016). Quantifying using mRNA levels has previously found a 15-fold increase in expression between HCC and background liver (Choi *et al.*, 2020). Patient age, gender and tumour size not been associated with Telomerase expression in this cohort, as previously reported in primary liver cancer (Zhou, Lu and Zhu, 2016).

Across the entire cohort, higher levels of tumour Telomerase has been consistently detected in patients without metabolic risk factors, (Allred, p= 0.007; H-score p= 0.004). This implies obesogenic states result in a metabolic disturbance do not utilise Telomerase in tumour tissues to the same extent as non-metabolically driven tumours.

When comparing all malignancies, tumours expressed more Telomerase than their paired background liver tissues, p<0.00006. There is evidence of CCAs preferentially expressing more Telomerase compared to HCCs (pairwise comparison p= 0.016). The correlation between Telomerase and non-metabolically associated tumours continues (p= 0.002) as well as a potential female gender related increase in protein expression (Allred score, p = 0.073). This gender bias was not reflected in tumour specific analyses, and has not been reported in the published literature. There was no difference in Telomerase expression when malignancies were grouped based on their promoter mutational/WT status, p= 0.306, consistent with previously published data from PLCs (Huang *et al.*, 2015).

Malignancies with Telomerase^{High} protein levels tended to be non-metabolic-related tumours (50.9%, 28/55) compared to just 18.2% (8/44) of patients with a metabolic risk factor, p= 0.001. Cholangiocarcinomas expressed higher levels of Telomerase (55.6%,

20/36) compared with mixed tumours (42.9%, 3/7) and HCCs (23.2%, 13/56), p= 0.005. There was a non-significant association between gender and high-expression of Telomerase which occurred in 48.6% (17/35) of women and only 29.7% (19/64) of men, p= 0.081.

The tumour-specific expression of Telomerase is also seen when CCAs are directly compared to HCCs (pairwise comparison, p= 0.032). However, only the CCA tumour subgroup expressed more Telomerase than their paired background liver using both the H-score, p< 0.0005, and Allred score, p< 0.0004. As supported by previous evidence of Telomerase activity in CCAs, with mRNA present in exfoliated needle biopsies (Itoi *et al.*, 2000).

High Telomerase-expressing CCAs were more often non-metabolically derived than metabolically driven, p= 0.001. Perhaps this is a cholangiocyte-specific example similar to previously published data, which used RNA sequencing to demonstrate that metabolic genes are downregulated in Telomerase^{High} expressing hepatocytes (Lin *et al.*, 2018).

There was anatomical variation in Telomerase expression, with more Telomerase in perihilar CCAs compared to their intrahepatic counterparts, p = 0.016. The mechanisms behind this site-specific increase in protein expression are unclear, but perhaps result from earlier biliary stasis and obstruction, which occurs earlier in the disease process for more proximal tumours. This would support data from Ozaki et al who studied Telomerase expression in 19 iCCA, five of these tumours which were due to hepatolithiases, and their control samples being benign hepatolithiasis-associated biliary tissues which occasionally expressed Telomerase (Ozaki *et al.*, 1999). Adding further weight to this is the fact that I have also found CCAs with Telomerase^{High} expression tend to be pCCA (100%, 6/6 in my study) compared to iCCA (only 14/30, 46.7%), p= 0.024.

Intrahepatic CCAs expressed more Telomerase than their paired background liver, p= 0.005. Intrahepatic CCA that developed in the absence of metabolic disease expressed more Telomerase than metabolically-associated iCCAs, regardless of the quantification method used in assessing staining (p= 0.001). This was confirmed when iCCAs were stratified into Telomerase$^{High/Low}$ expressers. Telomerase^{High} expressing tumours occurred in 75% (12/16) of the non-metabolic tumours, compared with just 14.3% (2/14) of the metabolic-associated iCCAs, p= 0.001.

When assessing my data in the whole cohort and tumour subgroups, I also did not detect any increase in Telomerase protein in pTERT^{Muts}. This implies my findings reflect what has already been reported in hepatocellular tumours, (Huang *et al.*, 2015).

5.1.5.2 Minor Findings

Two thirds of R1 CCA tumours expressed high levels of Telomerase, compared with one third of R0 tumours, p= 0.081. In this small study sample, this is perhaps early evidence of more malignant behaviour in Telomerase-expressing CCAs.

Further interesting data from the background liver tissues of CCA tumours has found a negative correlation between protein expression and patient age. Correlation coefficients between Allred score and patient age (coefficient = -0.417, p= 0.027) suggests younger patients who develop hepatic CCAs express more significantly more Telomerase in their native, non-malignant livers.

Surprisingly, when considering all HCCs, there was not preferential express tumour expression of Telomerase compared to background liver tissues (p= 0.051) which remained when FL-HCCs were excluded, p= 0.096. There was no difference in Telomerase expression in the promoter mutants, compared with WT, p= 0.820. There was no Survival benefit in Telomerase^{Low} expressing HCCs and no correlations with patient age, or tumour size.

Across all HCC subtypes, the well differentiated HCCs expressed higher Telomerase levels (4/9, 44.5%) compared with mild/moderately differentiated (8/37, 21.6%) and poorly differentiated tumours (0/6, 0.0%), but this was not a significant finding, p= 0.093. Only one third (10/30) of non-metabolic-driven HCCs expressed high Telomerase levels, compared to 11.5% (3/26) of metabolic related tumours, but this was also a non-significant finding, p= 0.065.

When fibrolamellar HCCs were excluded from analysis there was little change in the Telomerase expression findings. These HCCs failed to express significantly more Telomerase than their paired background liver tissues, H-score p= 0.438, Allred p= 0.096. There was also no difference in Telomerase expression between WT and promoter mutants, Allred p= 0.670, H-score p= 0.992.

High Telomerase expression in HCCs was not associated with adverse survival, patient age or tumour size. A potential difference finding higher Telomerase expression in one third (8/24) of non-metabolic, compared with 11.5% (3/26) of metabolic, tumours also failed to reach significance, p= 0.091.

Mixed CCA-HCC tumours shared more characteristics with HCCs than CCAs when Telomerase protein expression was considered. There was no difference in protein levels between tumour and background liver tissues, similar to the HCC group. There was also a positive correlation between patient age and Telomerase expression, again similar to the HCC cohort.

The positive correlation between patient age and Telomerase levels (Allred score: rho = 0.794, p= 0.033) in both mixed tumours and benign Adenomas (correlation coefficient = 0.750, p= 0.012) indicates that both of these tumour types preferentially express more Telomerase protein in older patients.

5.2 Discussion of Results

When attempting to put my findings within the current body of scientific knowledge, it is difficult to know how accurate my results are. Previously published works have found limited relationships between Telomerase expression and adverse clinical outcomes, or tumour characteristics (il Yu et al., 2017). Reassuringly, my work assessing Telomerase in liver tumours has not yielded many meaningful findings.

In addition to this: cumulative survival was not affected by Telomerase levels, similar to results from hepatocellular carcinoma, renal cancer, oral squamous carcinoma and cervical carcinoma (Carkic *et al.*, 2016; il Yu *et al.*, 2017; Yang *et al.*, 2017; Trifunovic *et al.*, 2018). The Kaplan Meier survival curves for the main tumour subtypes failed to demonstrate a difference in cumulative survival when tumours were stratified based on $Telomerase^{High/Low}$ expression.

It has been previously reported that epithelial cells with a high rate of self-renewal, such as those found in the GI tract, tend to express Telomerase protein and enzyme activity, albeit to a lesser extent than compared to tumour samples (Tahara *et al.*, 1999). The epithelial cells' ability to express Telomerase protein might confound my findings in this relatively small study. Cholangiocarcinoma can also express Telomerase mRNA in 85% of iCCA, as detected by in-situ hybridisation, indicating my findings replicate an alternative technique previously utilised on fresh tissue samples (Ozaki *et al.*, 1999). Transcriptional evidence of Telomerase activation is present in serum blood samples with hTERT mRNA detected in 84.5% of CCAs indicating that Telomerase activity is expected to be detected in the majority of CCAs (Leelawat *et al.*, 2006).

Telomerase promoter mutants are hepatocellular, not cholangiocellular, lineage specific genetic alterations (Quaas *et al.*, 2014). Elevated transcriptional activity due to $pTERT^{Muts}$ does not always correlate with promoter status. Previous multi-tumour

studies that found pTERT^{Muts} result in elevated Telomerase levels in both adult gliomas and thyroid cancers, but not HCCs, despite these tumours harbouring similar rates of promoter mutation (Huang et al., 2015; il Yu et al., 2017).

However, chronic inflammatory conditions within the liver, such as fibrosis and cirrhosis, have been reported to be associated with elevated Telomerase activity (Nault *et al.*, 2013; Lin *et al.*, 2018). My data failed to find similar increases (at least by protein expression) across the study cohort as a whole, or during the individual tumour analyses. Similarly, previously published work has found elevated Telomerase activity to correlate with advanced stage disease and poorer tumour differentiation in HCCs that was not reflected in my data (Zhou, Lu and Zhu, 2016).

When assessing Telomerase levels in both HCC and background liver samples, Choi *et al* used RT-PCR from curative resection specimens and reported a fifteen-fold increase in Telomerase expression in tumour tissues (Choi *et al.*, 2020). Early research found significant enzymatic activity, when quantified using the TRAP assay, in HCCs (Shimada *et al.*, 2000). My results, albeit using an alternative method of quantification, have failed to find any significant difference in Telomerase levels between HCCs (+/- FL-HCCs) and background liver tissues. This is perhaps due to differences in genomic and transcriptomic alterations that are not translated to altered proteomic expression, or perhaps as a result of a flawed scientific approach.

A key step in Telomerase-related carcinogenesis for HCCs is the nuclear translocation of the enzyme, which was the basis of using only nuclear positivity as a method for detection by the QuPath software package (Chen and Kong, 2010). I have been unable to reflect any of the previously published data by using IHC on archived FFPE samples as a proxy for either the TRAP assay or comparing with transcriptomic data quantified with PCR. However, evidence from the literature stating that the presence of Telomerase protein, as measured by Western blot, does not always correlate with

enzymatic activity detected by the TRAP assay (Tahara *et al.*, 1999). Clearly simple comparisons between quantification methods is an overly simplified approach, requiring further work to clarify this.

More recent work has also found varying rates of Telomerase protein and transcriptional material in HCCs. Using IHC to detect and quantify hTERT positive cells in 84.6% of HCCs with 78.2% of cases having detectable Telomerase mRNA, (Zhou, Lu and Zhu, 2016). These findings are of a similar magnitude, and as such, the differences between my findings and the published data are difficult to explain. Given that the only resource material available for this project is FFPE, meaning further work in fresh/frozen tumour samples would be required in a validation cohort.

Previous reports have demonstrated elevated levels of protein correlating with worsening differentiation in HCCs as well Telomerase being present in biliary epithelial cells (Kawakami *et al.*, 2000). Perhaps the reasons for lower detection of tumour Telomerase is due to QuPath's inability to characterise cytoplasmic staining. Notably, Zhou et al have stated that cytoplasmic Telomerase is more much more prominent in HCCs, whereas Huang et al feel it is detectable in both nuclear and cytoplasmic cellular compartments (Zhou, Lu and Zhu, 2016; Huang *et al.*, 2017). Whilst the nuclear staining appears to be very robust, there are many non-specific light shades of brown that – upon review of their figures - both Zhou and Huang feel should be regarded as positive staining.

More recently published works, albeit in renal cell cancer research, have acknowledged there is some cytoplasmic staining but have focussed on nuclear positivity when assessing hTERT expression in Renal cancers (Saeednejad Zanjani *et al.*, 2019). Indeed, Zanjani published an example of their four staining intensities (negative -, weak +, moderate ++ and strong +++) for hTERT protein detected during IHC in renal cell

cancer, as shown in figure 91, (Saeednejad Zanjani *et al.*, 2019). These are very similar to our staining intensities observed in the process of IHC optimisation.

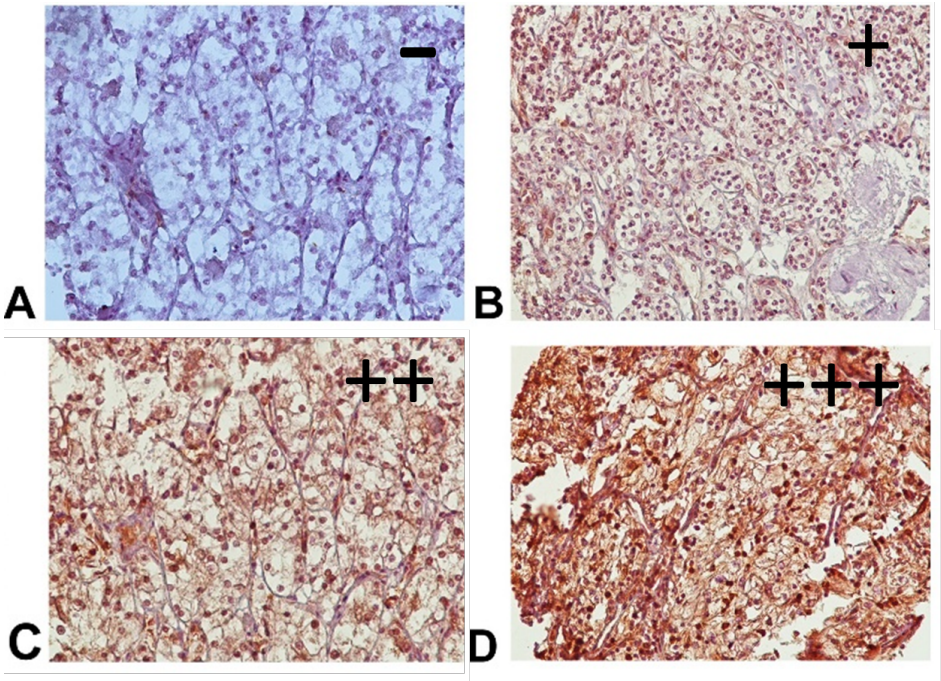


Figure 91. Assessing hTERT in renal cell cancer (RCC): negative staining (A), weak positive staining (+ B), moderate positivity (++, C) and strong positive staining (+++, D).

Whilst it is known that malignant cholangiocytes and hepatocytes express Telomerase activity, protein levels have not previously been compared between the two tumour types (Itoi *et al.*, 2000; Zhou, Lu and Zhu, 2016). However, Telomerase protein has been reported in both normal cholangiocytes and hepatocellular tumours (using IHC) so further work clarifying this expression is required (Kawakami *et al.*, 2000).

Given the reservations associated with IHC quantification of Telomerase protein mentioned in the literature, it would be very reassuring to have secondary-confirmation of my IHC-quantification findings, (Kim *et al.*, 2013).

5.2.1 Further Work

The most consistent finding from my work is increase in Telomerase expression in tumours that are not associated with metabolic conditions. Further exploration of exactly what happens to Telomerase expression in metabolic conditions (diabetes

mellitus, etc) may help to understand why these tumours express lower levels of Telomerase. Indeed, it may be a significant oversimplification to lump together all fat-related conditions, especially as fatty change in the liver is a common alteration due to hepatotoxic insults.

By greatly increasing the number of cases under study would be another way of confirming my putative correlations. The role of patient age has on Telomerase expression (as highlighted in the mixed and benign cohort) is one such example where greatly increasing these numbers could confirm this initial results. Another relatively simple, if not labour intensive, method would be to compare relative Telomerase expression in this dataset, with a secondary technique. This would give greater confidence in the validity of my findings. Repeating IHC using an alternative antibody, or using an alternative method (such as ISH) seems like a sensible place to start.

As mentioned above, there are a number of methods of inducing alterations in Telomerase expression – including promoter hypermethylation status studies using DNA bisulfite conversion and pyrosequencing, (Fotouhi *et al.*, 2019). Epigenetic changes (not promoter mutation) have been shown to result in increased Telomerase expression in small bowel neuroendocrine tumours. This approach used fresh/frozen samples but it could be attempted with FFPE-extracted DNA – as has been undertaken elsewhere, (Doyle, O'Riain and Appleton, 2011).

In addition to the epigenetic alterations, assessing viral DNA insertion as well as TERT amplification/ translocation events could help increase the understanding of Telomerase in PLCs. More recent work has also assessed the role of telomere length in aggressive liver tumours, with the exciting possibility of targeting these with anti-TERT antisense oligonucleotides as a targeted therapy, which has worked in cell culture and mouse models of HCC (Ningarhari *et al.*, 2020).

6 Ongoing and Future Work

Owing to time constraints and a global pandemic this section will outline my ongoing and planned future work for the haematological detection of universal tumour antigens.

6.1 ELISA Results

There ELISA results stalled at the optimisation stage and therefore failed to offer meaningful results for the whole cohort. The data is separately analysed for Survivin and Telomerase, as outlined below.

6.2 Survivin

As outlined in section **2.7.13** the calibration curves for Survivin ELISA were satisfactory, with a high R^2 giving confidence when estimating the results.

Diluting standards and samples with the manufacturers provided diluent gave the results shown in table 76. This includes samples at 1X and 0.1X (i.e., diluted 1:10 in diluent) to explore the accuracy of the assay. These results were somewhat disappointing as measurements could not be reliably made, given that the majority of samples lay in off scale concentrations (between the low standard [31.3pg/ml] and the zero-standard diluent) meaning these results were not within the reference range. Furthermore, there is also a paradoxical increase in OD when the serum is diluted - see 4E& 4F - between sample 45 (neat) and its 1:100 dilution (5A & 5B), table 76.

	Standard Concentration pg/ml					
	1	2	3	4	5	6
A	2000	2000	High	44 1:10	45 1:100	57
B	1000	1000	High	44 1:10	45 1:100	57
C	500	500	Medium	44 1:100	56	57 1:10
D	250	250	Medium	44 1:100	56	57 1:10
E	125	125	Low	45	56 1:10	57 1:100
F	62.5	62.5	Low	45	56 1:10	57 1:100
G	31.3	31.3	44	45 1:10	56 1:100	57 1:2
H	0	0	44	45 1:10	56 1:100	57 1:2

Survivin ELISA Results

	1	2	3	4	5	6
A	2.655	2.645	1.372	0.253	0.403	0.259
B	1.642	1.742	1.438	0.24	0.398	0.310
C	0.953	0.874	0.856	0.224	0.293	0.298
D	0.694	0.662	0.85	0.175	0.289	0.228
E	0.541	0.437	0.484	0.25	0.348	0.275
F	0.355	0.33	0.427	0.212	0.235	0.308
G	0.329	0.306	0.255	0.203	0.273	0.279
H	0.08	0.375	0.323	0.411	0.312	0.432

Table 76. Plate outline shown on the left panel with standards highlighted: yellow background, zero-standard shown with purple. Known High (red background), medium (orange) and low (green) concentrations of Survivin are also

present. Respective study numbers, and dilutions from 3G onwards. All reagents diluted with the provided diluent buffer. Right panel shows corrected results (OD 450-570nm) with red highlighting measurements that are between the low standard (31.3 pg/ml) and the zero standard, i.e., not on the scale for measurement.

Taken in combination, these results imply the ELISA kit is not able to detect human Survivin from serum samples with any confidence. The Survivin immunoassay control was only used in the first experiment as the recombinant protein, present in diluted porcine serum, was of limited value once we had established the assay was working.

To explore the serum-effect on this commercially available kit the experiment was repeated using 10% (v/v) healthy volunteer (HV) serum, using the provided diluent. These results can be seen in table 77.

	Standard pg/mL			Neat Serum	1:10 Serum
	1	2	3	4	5
A	2000	2000	HV 50	44	44
B	1000	1000	HV 50	44	44
C	500	500	HV 51	45	45
D	250	250	HV 51	45	45
E	125	125	HV 50 1:10	56	56
F	62.5	62.5	HV 50 1:10	56	56
G	31.3	31.3	HV 51 1:10	57	57
H	0	0	HV 51 1:10	57	57

Survivin ELISA Diluted in 10% HV Serum

	1	2	3	4	5
A	1.667	1.801	0.011	0.01	0.013
B	0.999	0.913	0.01	0.026	0.005
C	0.454	0.434	0.021	0.017	0.015
D	0.277	0.268	0.014	0.02	0.015
E	0.154	0.148	0.015	0.014	0.014
F	0.084	0.082	0.015	0.015	0.016
G	0.055	0.054	0.014	0.02	0.017
H	0.015	0.017	0.016	0.017	0.033

Table 77. Left panel shows standards highlighted on a yellow background with a zero standard on a purple background, and healthy volunteer (HV) compared with cancer patients' (numerical values) serum. Right panel: results highlighted with red font are between the low standard (31.3pg/ml, OD: 0.055) and the zero standard (0pg/ml, OD: 0.016).

Once the serum-effect was accounted for there were no detectable of Survivin in any of the samples tested. This is a somewhat disappointing result given that the ELISA kit used up precious biological samples and cost ~£500 for one plate. It is for these reasons, as well as time pressure, that an alternative direction was pursued.

6.3 Telomerase

The sandwich ELISA for Telomerase gave mixed results, as outlined below. The high standard (10ng/ml) Telomerase reading dipped compared to the 5ng/ml sample and was therefore excluded from the equation to estimate concentration from observed OD –

demonstrated by the lack of yellow-highlight in table 78. The provided diluent was used and giving the results from the ELISA are shown in table 78.

	Standard, ng/ml			Neat Serum	1:10 Dilution
	1	2	3	4	5
A	10.00	10.00	HV50	30	30
B	5.00	5.00	HV50	30	30
C	2.50	2.50	HV51	34	34
D	1.25	1.25	HV51	34	34
E	0.63	0.63	HV50 1:10	37	37
F	0.31	0.31	HV50 1:10	37	37
G	0.16	0.16	HV51 1:10	38	38
H	0.00	0.00	HV51 1:10	38	38

	Telomerase (ng/ml)				
	1	2	3	4	5
A	1.187	1.156	0.437	0.483	0.886
B	1.346	1.241	0.444	0.515	0.905
C	1.127	1.003	0.537	0.379	0.634
D	0.831	0.862	0.546	0.381	0.582
E	0.602	0.691	0.984	0.497	0.843
F	0.503	0.502	0.999	0.566	0.855
G	0.399	0.371	0.917	0.522	0.873
H	0.258	0.258	1.030	0.584	0.908

Table 78. Standard are highlighted (yellow background, zero-standard with purple. Plate layout is shown on the left sided table. The results (table on the right) appear to indicate an increase in the detected Telomerase with a 1:10 dilution – compare results from 4A&B with 5A&B.

There appears to be an increase in Telomerase detected in the 1:10 diluted samples, compared to physiological serum, similar to the Survivin ELISA results. This casts further doubt on the usefulness of this assay, and therefore severely limits the implications of these results.

Further to this, when attempting to explore the role human serum has on this assay, as show in table 79, data from the calibration standards run in 10% HV diluting serum was of exceedingly poor quality.

	OD's for 10% HV Serum used as diluent: Telomerase				
	1	2	3	4	5
A	1.268	1.519	0.384	0.325	1.103
B	1.511	1.406	0.368	0.347	1.135
C	1.464	1.204	0.472	0.209	0.853
D	1.331	1.245	0.716	0.24	0.719
E	1.138	1.287	1.214	0.248	0.841
F	1.096	1.206	1.618	0.273	0.846
G	1.153	1.445	1.510	0.241	0.784
H	1.618	1.799	1.627	0.358	0.872

Table 79. Using the same layout, and samples, as shown in table 78 – the effect 10% HV Serum has on the ELISA is shown here. Standard are highlighted on a yellow background with a purple background demonstrating the results from the zero-standard. The highest detected level in this ELISA was in the zero standard, meaning that further analysis was futile.

As shown in table 79, the highest values recorded were in the zero standard, making all of the corrected readings at 450nm negative values (standard OD (ng/ml) – diluent OD

(0ng/ml) = negative number). As a result of this, and the wide ranges seen in the calibration curve (figure 7) no further interpretation of these ELISA results were undertaken.

6.4 Discussion

For separate reasons both the Survivin and Telomerase ELISAs failed to yield any meaningful data. The Survivin assay failed to give any results above the low-standard's OD, making the interpretation of the respective concentration speculative, inaccurate and below the level of detection. Previously published work has used the same assay to successfully detect Survivin in serum from pancreatic cancer patients, so perhaps all of these HCC serum samples were negative for Survivin (Moazeni-Roodi et al., 2019). However, Jia et al, used an alternative ELISA kit and feel that Survivin is not a promising serological marker for HCC, which would agree with the findings from the limited sample of HCC patients that have been studied here (Moazeni-Roodi et al., 2019).

There were a couple of issues from the Telomerase assay. The inexplicable lowering of the detected OD from the 5ng/ml to the 10ng/ml standards and the increase in OD when detecting 0.1X compared to 1X sera make the interpretation of this assay troublesome. As a result, this measurement was excluded from the standard curve that may make higher Telomerase levels difficult to interpret. As the Survivin ELISA data was so poor, optimisation with the Telomerase ELISA experiments were undertaken using non-PLC serum samples. Serum from patients undergoing liver resection for metastatic colorectal cancer was used as these cases are more common than PLC resections, making the biological samples a slightly less precious research resource.

Colorectal cancer does indeed express Telomerase, so serum from these cases was deemed an appropriate substitute (Moazeni-Roodi et al., 2019). However, as serum

from PLC patients was not used, we are unable to comment on the role of circulating Telomerase levels as a potential biomarker, more that the Abexxa ELISA kit gave some unexpected results that should be repeated before any firm conclusions can be stated.

Antibody and contaminant molecule interactions (the so called 'matrix effect') can be ameliorated by a dilution step, as shown elsewhere in the published literature (Moazeni-Roodi et al., 2019). A 1:10 dilution of was undertaken in an attempt to reduce this error, as shown in table 78. To further investigate this would require more reagents and 'spiking' samples with a known concentration of Telomerase to fully understand whether or not these interactions are real or not (Moazeni-Roodi et al., 2019). This would, of course, require more reagents and use up precious samples but remains an avenue for future research to pursue. A repeat assay would also clarify the decrease in OD from 5ng/ml to 10ng/ml, but would require further reagents. As a result, this approach was not pursued further.

Diluting the Telomerase standards and samples in 10% HV serum was attempting to clarify whether or not there was a serum-effect in the sandwich ELISA. Sadly, this used up the last few wells, and did not yield any useful information.

To further research this, repeat experiments could be undertaken as a first step to ensure the results are reproducible. However, the ELISA results gathered to date have been of very limited use, and at quite a significant cost. It was therefore felt that the detection of circulating UTAAs would be better explored using an alternative method.

6.5 Exosome Results

An initial run failed to yield any results and was no immediately repeated because of the COVID-19 pandemic and subsequent societal lockdown. As can be seen from figure 92, the experiment appears to work with the calibrators (TERT and BIRC5) from cell line Huh7.5 being readily detected, and the exosomal housekeeping RNA (HPRT1) is also

detected. Regrettably, all of the samples processed failed to register a single positive result, all samples were run in duplicate.

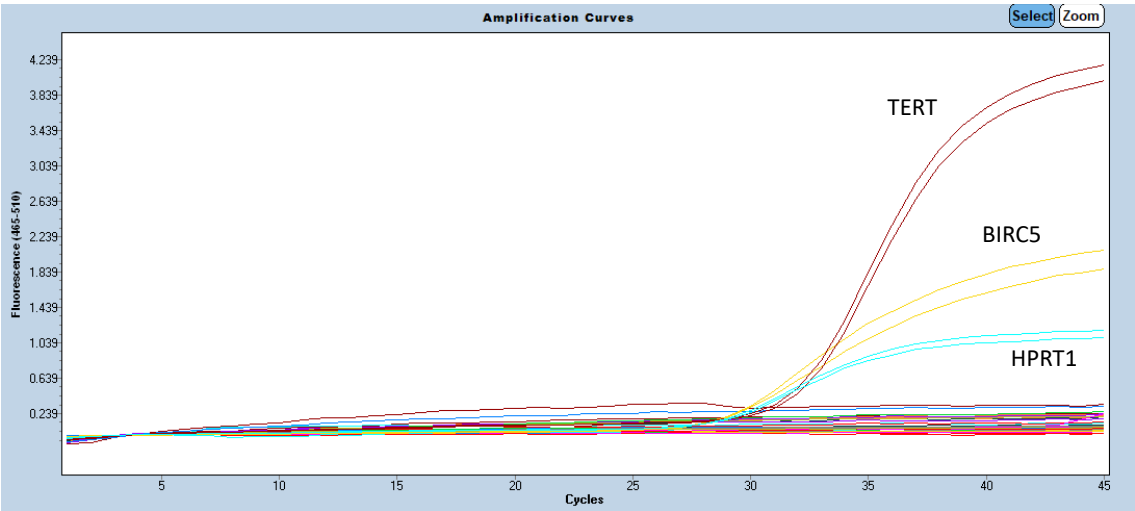


Figure 92. Quantitative PCR data from amplified Exosomal RNA. The calibrators (TERT and BIRC5) demonstrate the presence of this RNA from the Huh7.5 cell line and highlights that the reaction works. The internal control, HPRT1, is also detected.

This data comes from CRC serum samples, and is optimised, so could be repeated in blood from PLC patients.

6.6 Discussion

The internal control, HPRT1, and the calibrator, Huh7.5, have both worked very well indeed. It is very exciting to have a completely optimised protocol, with proof of principal for an assay to work. The next steps would include undertaking RNA extraction from exosomes derived from liver cancer, stable cirrhotics, and healthy volunteers to explore and describe these cohorts.

7 Discussion

7.1 Introduction

The aim of this project was to assess the role universal tumour antigens play in primary liver neoplasms, and how greater understanding of UTAAs and their role in tumour biology could be described in a UK cohort. Extensive work was been undertaken

internationally assessing these immortalising proteins, as well as alterations in the promoter regions, in both fresh/frozen and FFPE tumour samples.

Telomerase was hoped to be a druggable target for personalised therapies, yet initial results in this field have been disappointing. However, promoter mutants have been considered as a potential biomarker for a wide range of cancers, (Moazeni-Roodi et al., 2019). Similarly, Survivin's role in tumour biology has made it an attractive target for a wide range of anticancer-therapies (small molecule inhibitors, targeted RNA-degradation, inhibition of homodimerization and immunotherapies) regrettably with limited clinical effects, (in der Stroth et al., 2020). However, Survivin's role as a candidate biomarker requires ongoing research in a broad tumour population.

7.2 Challenges

Logistical challenges of commencing human-tumour research in a new site have limited the scope of this research project. Ethical approval took nine months, and as there was only formalin fixed tissues available for research, a more comprehensive analysis was not possible, as fresh/frozen tissues would have been required. However, usable DNA from archived tissues was able to be extracted and amplified to give promoter sequences. This labour-intensive approach required significant optimisation for both the pSurv and pTERT sequences, and is comparable with other reported data.

Protein expression of UTAA in FFPE tissues was also achieved, and quantified, for both the UTAA under study. The Survivin data appears robust, as crisp nuclear positivity was detected when a well-published primary antibody was used. Regrettably, the Telomerase antibody required additional significant optimisation, with extra blocking steps, and also required extra calibration for protein detection using the digital software package, QuPath. Interestingly, the Telomerase primary antibody has not been as extensively used in the literature as its Survivin counterpart.

When using digital pathology techniques to detect protein expression, true whole-tumour assessment was possible, giving highly detailed data on the whole-slide image and therefore a more accurate representation of protein expression by the liver tumours.

Significant challenges were met when the blood-work was undertaken to detect UTAA protein, and RNA levels. The commercially available ELISAs for both Survivin and Telomerase were not sensitive enough to detect any protein in the serum samples assessed. However, the exosomal work, whilst not yielding any results per se, includes both a calibrator and a positive control, and is an exciting prospect for future work as it appears to be fully optimised.

The clinical archive, and clinically-relevant data included in this study has some potential for improvement, which could alter the interpretation of my data. Gradations of fibrotic indices have not been used, as they are absent in the older material, meaning the binary presence/ absence has been used in its place. The same is true the survival data. Clinical timelines have simply not been recorded in a centralised database, meaning measures such as time to recurrence, disease-specific survival, overall survival, progression-free survival have not been compared, instead the somewhat clinically clumsy measure of overall survival was used. This has obvious implications on the validity of my correlations with the outcome data, given that the assumption has been made that the patient died of primary liver cancer, and not a secondary disease process.

The only way to avoid all of these biases would be to utilise a formal biobank where time has been taken to individually record this data. Regrettably this was beyond the scope of this research project, but is certainly achievable if more research resources were available locally.

However, the lessons I have learned as a principal investigator have proved to be invaluable, and will doubtless be of use in future research projects.

7.3 Promoter Mutations

Firstly, it is possible to use archived FFPE tissues for research purposes, and to sequence promoter regions (a known difficult target) which gives meaningful results that can be correlated with clinical parameters. The Survivin promoter SNP *rs9904341* has been proven to be a germline characteristic as there is concordance between background liver and tumour samples. Given that Survivin is re-expressed in malignancies, further qualifying the mechanisms behind this would be of scientific, and potentially clinical, benefit.

Across all neoplasms the GG promoter homozygotes tended to occur in patients who do not have liver cirrhosis, p= 0.042. When data from the pSurv and IHC results is combined in the CCA group, GG homozygotes express more Survivin protein than CC homozygotes, p= 0.048. Whilst C-dominance of the *rs9904341* SNP has been linked to increased cancer risk in studies based in Asia, there is no such link in Caucasian populations that have been studied (Moazeni-Roodi et al., 2019). In this British cohort of patients, I have early evidence that there may be a link between this GG genotype and adverse clinical outcomes, which warrants further research for clarification.

In all liver tumours, Telomerase promoter mutants have been shown to be associated with male gender (p= 0.001), liver fibrosis (p= 0.009), liver cirrhosis (p= 0.004) and HCCs, with 32.7% possessing a $pTERT^{Mut}$ (p= 0.002). In tumour sub-group analysis, there is a male preponderance (p= 0.005), an association with vascular invasion (p= 0.031), and possible evidence of shortened overall survival as $pTERT^{Mut}$ are associated with all-cause mortality (p= 0.043) as well as poorer cumulative survival, p= 0.083.

Globally, $pTERT^{Muts}$ have been reported to occur in 43.9% (1831/4170) of HCCs, (in der Stroth et al., 2020). My data has achieved comparable rates, but not recreated these

findings, which have been quoted as being present in over 60% of HCCs in a European cohort, (Nault & Zucman-Rossi, 2016).

There is also evidence of both intra-tumoural and inter-tumoural heterogeneity in the $pTERT^{Muts}$ in HCCs, which may benefit from further work using either FFPE or fresh/frozen materials. Whether these specific driver mutations result in clonal expansion within a particular tumour has not been proven in my results, assuming the methods I have used are completely accurate. Cross-validation with a second technique (ddPCR, or a KASP assay) could confirm these results (Hubáček *et al.*, 2015; McEvoy *et al.*, 2017).

The HCC tumour-specificity of $pTERT^{Muts}$ has previously been reported, with only 1.9% of all iCCAs sequenced possessing this genetic alteration, (Quaas *et al.*, 2014; in der Stroth *et al.*, 2020). Of the 362 iCCAs that have been sequenced (and published) only 7 have contained a $pTERT^{Mut}$ (in der Stroth et al., 2020). My work in sequencing DNA from 30 iCCAs has contributed to this field, and also confirmed previous results that more distal tumours (pCCA, n= 6 in my study with n= 86 in Nakamura et al) also do not harbour $pTERT^{Muts}$ (Nakamura *et al.*, 2015).

7.4 Survivin Expression

Using digital pathology methods to detect Survivin immunohistochemical expression in liver tumours has been successfully demonstrated. Each of the main tumour subtypes under study have given subtly different results which, should they be validated in a larger cohort, could be used as a clinically-relevant adjunct in routine patient care. Survivin has demonstrated greater expression in liver tumours (both benign and malignant) highlighting its role in tumourigenesis.

Larger malignancies have been shown to express more Survivin ($p= 0.046$) and there is an association with death ($p= 0.016$), advanced tumour stage ($p= 0.047$) and vascular

invasion (p= 0.008). Tumours that highly express Survivin are associated with death (p= 0.007), are of a more advanced stage (p= 0.007), and have invaded the vasculature (p= 0.001) despite being more most expressed in FL-HCCs (p= 0.018). Cumulative survival is also reduced in malignancies that express high levels of Survivin protein, p= 0.001.

Liver tumour subtypes behave differently, with advanced HCC tumour grades expressing more Survivin (p= 0.007) as well as correlating with vascular invasion (p= 0.013). Even assessing the background liver in HCC patients could be of benefit, with differential Survivin expression depending on the tumour grade that subsequently grow, p= 0.042. The Survivin promoter SNPs studied showed those homozygous for GG express more Survivin in Cholangiocarcinoma, p= 0.048. There are also clinical correlations between CCA and death (p= 0.005), advanced stage disease (p= 0.024) and perineurally invasive tumours (p= 0.045). Cumulative survival in CCA reduced in tumours that express high levels of Survivin (p= 0.002), as is tumour stage (p= 0.021) and GG homozygosity (p= 0.021).

These results could be translated to clinical medicine and used as an aide when assessing higher-risk liver tumours.

7.5 Telomerase Expression

Telomerase expression in liver tumours has brought mixed results, and raised more questions than providing answers. Background liver Telomerase expression is present in all liver tissues, meaning using this method to differentiate tumour from background liver is not appropriate. In the malignant tumours, pairwise comparisons between tumours types was able to demonstrate differences in Telomerase expression between HCCs and CCAs, p= 0.032. Tumours with Telomerase promoter mutations do not express more Telomerase in any cohort, or subgroup analysed.

Somewhat oddly, non-metabolically driven malignancies expressed more Telomerase, p= 0.004. The reasons behind this require further work as they may be an artefactual finding in an overly simplified model of liver tumour aetiology. Non-metabolically derived CCA express more Telomerase (p= 0.001) as do perihilar CCAs (p= 0.016). Cumulative survival did not differ between patients whose tumours expressed high or low Telomerase levels, meaning this would be a poor method of stratifying high-risk patients.

Both the mixed tumours (p= 0.033), and the benign adenomas (p= 0.012) have an apparent correlation between patient age and Telomerase expression. Further work to ascertain why these particular tumours behave like this is required, especially as $pTERT^{Muts}$ status is clearly not involved. This could include assessment of the role of TERT amplification/ translocation or (less relevant in this study population) genetic insertion events in the context of HBV, (Nault *et al.*, 2019).

Overall assessing Telomerase protein expression has not yielded many meaningful results in this study, especially given the concerns previously raised as to the validity of Telomerase quantification by IHC, (Kim et al., 2013).

7.6 Haematological Detection

Haematological expression of UTA has not proven helpful in assessing patients with hepatic neoplasms. The ELISA experiments have failed to produce data of a quantity required to comment on the role of Survivin or Telomerase in liver neoplasms, as has previously been reported when using Telomerase quantification in lung cancer (Targowski et al., 2010). Of note previous research in HCCs failed to find a role for serological detection of Survivin, (Jia *et al.*, 2015).

The scientific approach has yielded some very promising early results in the optimisation of the exosomal quantification work, but not yet enough to correlate with

clinical or research parameters, including tissue expression or promoter variants that have been assessed in this project.

7.7 Bias and Statistics

During this research project every effort has been made to reduce any inherent bias that may alter the results. However, the specimens that have been studied are all from patients who were medically fit enough to undergo major abdominal surgery, meaning an inherent selection bias that is out with my control. The only way to ensure the results demonstrated above are accurate in all liver tumours would be to include biopsy specimens, thereby representing the patient cohort more accurately. The volume of tumour tissue from needle-biopsy specimens is sometimes so limited that this is deemed insufficient for a formal medical diagnosis, meaning inclusion into research projects would not be possible. A potential way around this issue would include second-pass biopsies (which further increase the risk of morbidity and mortality to the patient) for future research projects.

Numerous analyses have been made on the same data presented above with some corrections for repeated statistical tests, i.e., Bonferroni correction. However, a formal assessment of possible false-discovery rates has not been undertaken when assessing the neoplastic/malignant/ tumour subgroup analyses. This means these results and possible inferences on clinical practice should be interpreted/ acted upon with caution.

7.8 Conclusion

In conclusion, I have used a variety of techniques to explore the role of UTAA in primary liver neoplasms. Noteworthy highlights include the successful Sanger sequencing of promoter regions from DNA extracted from FFPE tissues, and the use of a digital pathology platform to quantify protein expression of UTAA in PLCs – which correlated with clinical parameters. As my sequencing results provided evidence of

pTERT^{Muts} being an HCC-lineage specific genetic alteration, sadly the IHC for Telomerase was quite disappointing. Whilst the Survivin sequencing data failed to yield any meaningful results, the IHC results demonstrated significant correlations, some of which are novel.

However, the lack of progress on my biobanked blood samples is a source of frustration, despite numerous attempts at protein quantification using an ELISA. The Exosomal work is very promising and may yield meaningful results if fully pursued.

Being the first researcher in the South West of England to explore a new cohort of patients has been a real privilege. Progress through the original ethics application has provided me with an invaluable set of transferable skills for my future research activities. Being involved in the embryonic stages of biobank recruitment has also been of significant benefit, and has given me a new perspective on the effort involved in patient identification and recruitment.

8 Future Work

Immediate future work will include using an optimised protocol on serum samples taken from patients with primary liver diseases, including cirrhosis and cancer, using the real time quantitative PCR method. Assessing the RNA content in the exosomal fraction of blood from these patients will help clarify whether the role of UTAA in PLC.

However, alternative methods could also be used such as the relative quantification of serum protein using mass spectrometry. Recent work has used liquid chromatography mass spectroscopy (LC/MS) in a cell culture model of HCC, using hTERT knockdown (siRNA) methods (Choi *et al.*, 2020). Similar approaches have described the role of Survivin in CD4+ T cells play in patients with HIV (Kuo et al., 2018). One of the many benefits of using LC/MS over individual protein detection methods is the sheer volume

of data that could be generated from a few samples. Not only would I be able to ascertain whether Survivin and/or Telomerase are detected in blood samples, but I would be able to study numerous targets simultaneously, including other enzymes/ proteins involved in their respective signalling pathways.

The Telomerase enzymatic action has only been determined by using the TRAP assay, which requires fresh/frozen tissue samples. Given unlimited resources, it would have been beneficial to attempt this assay in tumour samples from patients to better understand the role of Telomerase in primary liver cancers. Further work on understanding the role of Telomerase in PLCs could also assess the other methods (currently known) that can result in Telomerase activation: TERT amplification, TERT translocation and viral insertion into the TERT gene, (Nault *et al.*, 2019). Epigenetic changes (such as promoter methylation) could also be explored in my research cohort, as has been demonstrated in neuroendocrine tumours of the small bowel, (Fotouhi *et al.*, 2019).

My data has shown Survivin to be a potentially useful biomarker in PLCs. Stratifying tumours by Survivin protein expression may be a useful method of identifying higher-risk cases, but this would need to be supported by a larger study cohort. Whether liver tumours shed peripherally detectable Survivin, to act as a haematological biomarker, also warrants further assessment.

The methylation status of the Survivin promoter, and correlations with Survivin expression (both transcribed and proteomic) has given mixed results in a variety of cancers (Lyu *et al.*, 2018). Assessing the methylation of the Survivin promoter in liver cancers, and correlating this with the results presented here would be a first for liver cancer. Alternative methods of assessing the epigenetic control of Survivin expression could also include the quantification of microRNAs, and how these compare with proteomic Survivin (Rahban *et al.*, 2019).

In summary there are many possible avenues to pursue when considering how to expand on the work presented in this thesis, when considering the role of UTAA in liver tumours.

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