Molecular Mechanisms in the Pathogenesis of Hepatoblastoma

Rachel Purcell

University of Otago
Abstract

Hepatoblastoma accounts for 1% of new cancer diagnoses in childhood and is the most common childhood liver cancer. While most cases of hepatoblastoma (HB) are sporadic and its aetiology is unknown, there is a close association of HB with developmental syndromes such as the Beckwith-Wiedemann Syndrome (BWS) and Familial Adenomatous Polyposis (FAP). Clinical staging and histologic classification are complex and the current approach to risk stratification of these patients is based on clinical features such as the tumour stage, and the extent of liver involvement. Although treatment of HB with platinum-based chemotherapy followed by resection is highly successful there remains >15% of HB that suffer from relapse. These relapse patients are often refractive to conventional chemotherapy and have a poor prognosis.

The International Society of Paediatric Oncology liver tumour strategy group (SIOPEL) has conducted a series of clinical trials, with the aim of improving the overall clinical outcome of HB. In this study we accessed 94 formalin-fixed paraffin-embedded (FFPE) tumour samples from patients registered with SIOPEL. Corresponding clinical, histologic and survival data was available for all samples. We created a tissue microarray from these samples and examined the expression of E-cadherin, Cyclin D1, Ki67 AFP and β-catenin using immunohistochemistry. Statistical analysis revealed that Cyclin D1 and Ki67 expression were significantly associated with outcome in our HB cohort. This has revealed two potential biomarkers that may aid in prognostication.

Activation of β-catenin is a hallmark of hepatoblastoma and appears to play a crucial role in its pathogenesis. However, while aberrant accumulation of β-catenin is a common event in HB, the high frequency of protein expression cannot always be accounted for by mutations or deletions in CTNNB1, the β-catenin gene. 87% of this HB cohort showed aberrant accumulation of the β-catenin protein but only 15% had mutations in the β-catenin gene by sequence analysis. In this study we also investigated alternative activation of β-catenin by HGF/c-MET signalling in our large cohort of HB patients. Activation of the HGF/c-Met pathway leads to tyrosine
phosphorylation on residue 654 of β-catenin resulting in nuclear localization and downstream signalling. Immunohistochemical analysis revealed a large subset of HB, 83%, with cytoplasmic localization of tyrosine654-phosphorylated β-catenin with 30% showing additional nuclear accumulation. Statistical analysis revealed an association between nuclear expression of c-MET activated β-catenin and wild type CTNNB1. Our analysis identifies a significant subset of hepatoblastoma patients for whom targeting of the c-MET pathway with TRK inhibitors may be a treatment option.

To investigate potential epigenetic regulation of HB pathogenesis we examined microRNA profiles from a number of tumours using microarrays. No differential expression was found between wild type and mutant CTNNB1 tumours or between relapse and non-relapse patients. However particular miRNAs appear to be upregulated/down-regulated in HB when compared to published data on normal liver miRNA profiles, including miRNAs known to be involved in cancer.
Acknowledgements

First and foremost I offer my sincerest gratitude to my supervisors, Dr Michael Sullivan and Dr Tracy Hale, who have supported me throughout my studies with patience and knowledge whilst allowing me the scope to work in my own direction. I am especially grateful to my convenor, Dr Martin Kennedy, whose encouragement, guidance and support from the initial to the final stages of my studies went above and beyond the call of duty.

I am also grateful to my thesis advisors, Dr Tony Reeve and Dr Antony Braithwaite for their input and advise. My thanks also to Michelle Cheale from anatomical pathology for assistance with immunohistochemistry and to Dr Carina Miles and Dr Clinton Turner for spending so much time analysing the slides. Thanks to Howard Potter and Andrew Fellowes from Molecular Pathology for all the help with sequencing.

I am indebted to Dr Rudolf Maibach and Dr John Pearson for statistical analysis of my results and help with planning experiments and to Les McNoe and Dr Mik Black for guidance with microarrays.

Thanks to Grace and Lucy, Frank, Rosie, Timmy and George for providing a welcome distraction, lots of laughter and joy and for keeping me grounded. Finally, to Tony for all the love and support over the years. I couldn’t have done it without you.
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<th>Definition</th>
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<tbody>
<tr>
<td>AFP</td>
<td>Alpha-fetoprotein</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens junctions</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BWS</td>
<td>Beckwith-Wiedemann syndrome</td>
</tr>
<tr>
<td>CDDP</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf Intestinal Alkaline Phosphatase</td>
</tr>
<tr>
<td>CKI</td>
<td>Casein Kinase</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphoblastic leukemia</td>
</tr>
<tr>
<td>COG</td>
<td>Children’s Oncology Group</td>
</tr>
<tr>
<td>COSMIC</td>
<td>Catalogue of mutations in Cancer</td>
</tr>
<tr>
<td>D-MEM</td>
<td>Dulbecco’s Minimum Essential Media</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Dvl</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine-tetra-acetic acid</td>
</tr>
<tr>
<td>EFS</td>
<td>Event-free survival</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-Mesenchymal transition</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FE</td>
<td>Feature Extraction</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
</tr>
<tr>
<td>Fz</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GPOH</td>
<td>German Society for Paediatric Oncology and Haematology</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>H+E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HB</td>
<td>Hepatoblastoma</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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</tbody>
</table>
HIER  Heat-induced epitope-retrieval
HR    High Risk
HRP   Horse Radish Peroxidase
IHC   Immunohistochemistry
JCRB  Japanese collection of research bioresources
LBW   Low birth weight
LRP   Lipoprotein receptor related protein
MEM   Minimum essential media
miRNAs MicroRNAs
OS    Overall survival
PBS   Phosphate buffered saline
PCV   Packed-cell volume
pen/strep Penicillin/streptomycin
PLADO Cisplatin and Doxorubicin
PRETEXT pretreatment extent of disease
pri-miRNAs Primary miRNAs
QC    Quality control
RISC  RNA-induced silencing complex
RT-PCR Reverse-transcription polymerase chain reaction
SCUD  Small-cell undifferentiated
SF    Scatter factor
SHH   Sonic hedgehog
SIOP  International Society of Pediatric Oncology
SIOPEL SIOP liver tumor strategy group
SR    Standard risk
SuperPLADO Carboplatin, Doxorubicin and Cisplatin
TBE   Tris-borate EDTA
TBS   Tris buffered saline
TMA   Tissue Micro-Array
TRK   Tyrosine receptor kinase
VLBW  Very low birth weight
Y654-β-catenin Tyrosine 654-phosphorylated β-catenin
WT    Wild type
Chapter One
Introduction and Literature Review
Chapter 1: Introduction and literature review

1.1 Introduction
Paediatric embryonal tumours are a heterogenous group of solid tumours usually presenting in young children. These types of cancers differ from adult cancers, which are predominantly carcinomas. Solid tumours account for about half of childhood cancers with leukaemias and lymphomas combined accounting for the other 50%. Central nervous system cancers are the most predominant of the solid tumours (20%), followed by tumours of the sympathetic nervous system (neuroblastoma), soft tissue sarcomas (rhabdo- and leio-myosarcoma), kidney tumours (Wilm’s tumour), bone tumours (osteosarcoma, Ewing’s Sarcoma) and other rarer malignancies such as hepatoblastoma of the liver making up the rest.

Histologically, the tumours often look very similar to embryonal tissue of the organ or tissue of origin. Gene expression profiling has also shown a close relationship between embryonal tumours and the immature cells from the organ of origin at a molecular level, and this provides evidence that these tumours arise as a result of corrupted development. Although sometimes associated with specific syndromes, most of these types of cancers are sporadic. That is to say they are not inherited and as the majority of these cancers arise very soon after birth, and indeed some are detected in utero, environmental causes are unlikely. Therefore it is not possible to prevent embryonal tumours of childhood and treatment by chemotherapy and/or surgery is necessary for survival. Most mainstream chemotherapy used to treat adult cancers targets proliferating cells and these types of treatments have severe consequences for immature developing organs in children. The current emphasis must be on effective targeted treatment with minimal side effects to improve survival and quality of life for children with these tumours.

To improve treatment options it is imperative to understand the molecular pathogenesis of the tumours. Many of the same signalling pathways seen in normal embryonic organ development e.g. Wnt/β-catenin and Sonic Hedgehog (SHH), are
aberrantly activated in paediatric tumours and it is very likely that unchecked signalling contributes to tumour development. As epigenetic regulation by microRNAs plays such an important role in organ development, mis-regulation of microRNA expression may also contribute to embryonal tumour development. Understanding the underlying biology of embryonal tumours may uncover novel therapeutic targets for use in the clinical setting. In this study we use hepatoblastoma as a model tumour to study mechanisms involved in embryonal tumour pathogenesis.

1.2 The normal liver
The liver is a vital organ that has a wide range of functions in the body. It is involved in carbohydrate, protein and lipid metabolism. The liver also produces coagulation factors, hormones and albumin and it is involved in detoxification and storage of vitamins and glucose (Pardi and Cetin, 2006). In the first trimester the foetal liver is responsible for red blood cell production (Campagnoli et al., 2001). The liver is mainly made up of two types of cell; hepatocytes and cholangiocytes and these are thought to arise from a bipotential precursor cell (Tanaka et al.)

1.3 Hepatoblastoma
Hepatoblastoma (HB) is a rare tumour and although it is the most common liver cancer in children, it accounts for only ~1% of all childhood malignancies. It is most common in the first five years of life and the incidence decreases with age. In recent times there has been a steady increase in the occurrence of reported HB cases and this is thought to be due to the improved survival of low-birth-weight babies HB usually presents as an abdominal mass and is often asymptomatic. HB can occur as part of Beckwith-Wiedemann syndrome (BWS) and has also been reported in cases of familial adenomatous polyposis (FAP) syndromes although most cases are sporadic. Clinical staging and histologic classification are complex and the current approach to risk stratification of these patients is based on clinical features such as the tumour stage and the extent of liver involvement.

1.3.1 Epidemiology
The incidence of hepatoblastoma is 0.7-1/1 million population per year in children in Western countries with approximately 100 new patients presenting in the United
States each year (Darbari et al., 2003, Pritchard et al., 2000). Hepatoblastoma occurs 4-5 times more frequently in white children than in black children. Male-to-female predominance, which is 2:1, is more apparent in younger children (Weinberg and Finegold, 1983). In children older than 5 years, the sex difference disappears. More than 90% cases are seen in infants and children younger than 5 years, with most occurrences (66%) prior to age 2 years (Darbari et al., 2003, Stocker, 1994).

1.3.2 Low birth weight and HB
Low birth weight (LBW 1.5 -2.5kg) and very-low birth weight (VLBW <1.5kg) are disproportionately at risk from developing HB (Slovis and Roebuck, 2006). The largest study to date found that VLBW babies are more than 50 times more likely to develop the disease than babies born within the normal range (Reynolds et al., 2004). VLBW and LBW infants account for under 10% of live births in Western society but up to 40% of HB occur in these groups (Ribons and Slovis, 1998). The use of neonatal oxygen has been linked with increased cancer risk in children (Spector et al., 2005) and the Children’s Oncology Group (COG) is currently undertaking a large study to investigate possible causes for the high levels of HB linked with birth weight. It has been hypothesized that “catch-up growth” in LBW babies may lead to aberrantly increased levels of growth factors such as hepatocyte growth factor (HGF), which may contribute to tumour growth.

1.3.3 Syndromes associated with HB
Hepatoblastoma has been reported in association with Beckwith-Wiedemann syndrome and familial adenomatous polyposis syndrome (FAP). BWS is an overgrowth syndrome with increased risk of cancer (Elliott et al., 1994). It is linked to deregulation of the 11p15 chromosomal region although specific causative genetic or epigenetic defects have not yet been identified (Ping et al., 1989). Children with BWS have a 600-fold increased risk of developing HB (DeBaun and Tucker, 1998). FAP is an inherited disorder characterized by the growth of intestinal polyps, ultimately resulting in colon cancer. FAP is associated with truncating mutations in the adenomatous polyposis coli (APC) gene leading to activation of the Wnt signaling effector protein, β-catenin (Kinzler et al., 1991). The risk of developing HB is 800-times higher in children with a family history of FAP (Giardiello et al., 1991).
1.3.4 Clinical Features
Hepatoblastoma is usually diagnosed as an asymptomatic abdominal mass with an average diameter of 10-12 cm at diagnosis. The tumour most often is a unifocal, well-circumscribed mass, but it may be multinodular. The right lobe is more commonly affected although both lobes may be involved if the tumour is multinodular (Exelby et al., 1975). Radiological findings from ultrasound and x-ray computed tomography scans are used extensively to stage the disease.

On gross inspection, the epithelial type tends to be homogenous, while mixed epithelial-mesenchymal tumours demonstrate a more variegated appearance with areas of osteoid, cartilage, calcification, fibrosis, necrosis, and haemorrhage that reflect the mesenchymal components. The small-cell undifferentiated (SCUD) variant frequently contains a large focus of central necrosis. Microscopic vascular invasion may be seen beyond an apparently encapsulated. Lung metastases are present in 10% of patients on diagnosis and portal vein involvement is occasionally seen. Bone metastasis is a rare finding at diagnosis.

Alpha-fetoprotein (AFP) levels are usually increased upon diagnosis. However, very low AFP <100ng/ml, or very high levels, >1,000,000ng/ml have been linked with poor outcome and these patients are treated as high risk (De Ioris et al., 2008, von Schweinitz et al., 1997b). AFP is also used to monitor the response to chemotherapy and identify patients who are poor responders and should be considered for alternative therapy (Van Tornout et al., 1997, Kubota et al., 2004).

1.3.5 Histopathology
Classification of primary paediatric neoplasms has evolved over the past 50 years with morphologic distinction between HCC and HB being a major breakthrough (Weinberg and Finegold, 1983). Many histologic subtypes of hepatoblastoma exist arising from the varied cell types within the tumour. Approximately 56% of tumours are of epithelial origin, which is subclassified further as pure fetal (31%), embryonal (19%), macrotrabecular (3%), and small-cell undifferentiated (anaplastic) (3%). Fetal
type HB resembles hepatic cells in the normal pre-natal liver while embryonal cells are less differentiated (Ishak and Glunz, 1967, Stocker, 1994). Macrotrabecular subtype of wholly epithelial HB consists of fetal or embryonal cells arrayed in trabeculae and are morphologically more mature (Zimmermann, 2005, Gonzalez-Crussi et al., 1982). The cholangioblastic subtype contains bile ducts or duct-like features that may reflect the maturation of the tumour but the biologic significance is unknown (Zimmermann, 2002). The small cell undifferentiated subtype does not fit into the classification as easily. It is similar to small-cell blue tumours such as neuroblastoma. It was formerly termed anaplastic (Haas et al., 1989, Kasai and Watanabe, 1970). The source of SCUD cells is unknown but it has been proposed that they may arise from hepatic stem cells (Ruck et al., 1997).

Approximately 44% of tumours contain both mixed epithelial and primitive mesenchymal components and histologic patterns frequently co-exist (Stocker, 1994, Weinberg and Finegold, 1983). Mesenchymal elements may consist of osteoid, chondroid, or myofibroblastic features with an increase in osteoid common after chemotherapy (Saxena et al., 1993, Stocker, 1994). Mixed tumours may also express teratoid features such as cartilage, bone or muscle (Conran et al., 1992). The classification of HB is a work in progress with constant refinement being reported.

The complex histology and rarity of the tumour make it difficult to classify for the general pathologist. Incorporation of HBs into clinical trials allows review by a central pathologist with more extensive experience and this has aided in subtype classification. The current classification distinguishes between the different phases of liver cell development and differentiation and recommends classifying as described in Table 1.1 (Stocker, 1994, Zimmermann, 2005, Rowland, 2002).

1.3.6 Histologic Classification and outcome
Correlation between histologic type and disease outcome in HB has been reported as early as 1970 (Kasai and Watanabe, 1970). This study reported an association between pure fetal HB and improved survival and this result has been reflected in subsequent studies (Haas et al., 1989, Lack et al., 1982, von Schweinitz et al., 1997b, Weinberg and Finegold, 1983). However, no correlation between fetal subtype and
outcome was reported by Conran et al or in a more recent report from the SIOPEL-1 study (Brown et al., 2000, Conran et al., 1992). The rare macrotrabecular subtype responds differently to chemotherapy than fetal or embryonal subtypes and conflicting results regarding prognosis have been reported from small trials (Brown et al., 2000, Conran et al., 1992, Haas et al., 1989, Zimmermann, 2005). There have also been conflicting reports on the prognostic significance of stromal elements in HB (Haas et al., 1989, Heifetz et al., 1997). However, there is agreement in the literature about the poor prognosis for SCUD subtype (Lack et al., 1982, Haas et al., 1989, Ortega et al., 2000). A recent study by Meyers et al reported SCUD histology as an important factor in determining survival in HB (Meyers et al., 2009). All eleven patients with SCUD histology in unresectable tumours died of disease in a separate study (Trobaugh-Lotrario et al., 2009). Conflicting reports about the prognostic significance of subtypes may be in part due to different sampling, staging systems and treatment in different groups and this underlines the need for international cooperation.

<table>
<thead>
<tr>
<th>Table 1.1: Current classification of HB</th>
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<tr>
<td><strong>Histologic Type</strong></td>
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</tr>
<tr>
<td><strong>Wholly epithelial type</strong></td>
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<tr>
<td><strong>Mixed epithelial and mesenchymal type</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td><strong>Hepatoblastoma, not otherwise stated</strong></td>
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</table>

1.3.7 Cytogenetic analysis of HB

Many cytogenetic studies have been carried out on small numbers of HB tumours over the last 15 years. Common findings reported were trisomy 20, trisomy 2, trisomy
8 and unbalanced translocations of chromosomes 1 and 4 (Ma et al., 2000, Schneider et al., 1997, Sainati et al., 1998). Due to the lack of numbers in these studies it was impossible to determine the rate of cytogenetic abnormalities in HB. A large study involving 111 cases of HB reported chromosomal abnormalities in 50% of their cohort with similar structural and numerical abnormalities as reported in the previous studies (Tomlinson et al., 2005). Neither this study nor another study by Von Schweinitz et al of 56 HB patients, found any association of genetic alteration with outcome (von Schweinitz et al., 2002).

1.3.8 Gene expression profiling of HB tumours
The first analysis of gene expression profiles in HB was carried out in 2003 (Nagata et al., 2003). This study found a set of twenty-six predictor genes that were differentially expressed between tumour and adjacent normal tissue, including some genes involved in regulation of cell division and tumour cell growth. The PLK1 oncogene was found to be expressed at higher levels in tumour versus normal tissue in another study, with high expression correlating with poor outcome (Yamada et al., 2004). An in-depth study of gene expression, chromosomal abnormalities and histologic features in a large number of HB tumours found that a sixteen gene-signature could distinguish aggressive tumours with statistical significance and this signature also classified the tumours on the basis of their underlying biology (Cairo et al., 2008).

1.4 International Hepatoblastoma study groups: Treatment and Staging of HB
The relative rarity of hepatoblastomas means that very few cases are seen by any one center. This means that co-operative studies have been necessary to assess treatment strategies, and aid in prognostication and staging. The main study groups are centred in Europe, North America, Germany and Japan.

1.4.1 SIOPEL
The International Society of Paediatric Oncology liver tumour strategy group (SIOPEL) was founded in 1987 under the umbrella of the International Society of Paediatric Oncology (SIOP). The main goals of SIOPEL are the following:
• To develop comprehensive clinical research programs on childhood hepatoblastoma and hepatocellular carcinoma.
• To promote basic and translation research in this field, making available biological material derived from these rare patients.
• To promote worldwide cooperation.
• To develop research programs for treating childhood liver tumours in countries with limited economic resources.
• To develop models for running international research on rare tumours.

1.4.1.1 SIOP LE-1 clinical trial
SIOP LE has conducted a series of clinical trials, SIOP LE 1-3, with the aim of improving the overall clinical outcome of children with Hepatoblastoma (HB) or Hepatocellular Carcinoma (HCC). Following the success by SIOP at treating Wilm’s tumours with preoperative chemotherapy followed by surgery, it was decided to adopt a similar strategy with HB (Lemerle et al., 1983). In the first SIOP LE study, patients with HB or HCC were treated with 4-6 courses of chemotherapy, Cisplatin and Doxorubicin (PLADO), before surgical resection of the primary tumour was attempted. SIOP LE devised a preoperative system of staging based on radiological imaging techniques. Primary chemotherapy rather than surgery has been the mainstay of treatment within SIOP LE. This treatment aims to shrink the tumour size and allow for complete resection, which is necessary for survival from HB. Using the data collected in SIOP LE-1 it was possible to identify various prognostic factors for patients with HB. Lung metastases at diagnosis and PRETEXT (pretreatment extent of disease) stage were statistically significant factors associated with 3 year overall survival (Perilongo et al., 2000).

1.4.1.2 PRETEXT staging system
The PRETEXT was introduced as part of the first SIOP LE prospective trial and has been used since to stratify paediatric liver tumours (Brown et al., 2000, Schnater et al., 2002, Aronson et al., 2005). PRETEXT is based on division of the liver into four sections and the PRETEXT number was derived by subtracting the highest number of contiguous liver sections without disease from four as shown in Table 1.2 (Roebuck et al., 2006). Additionally the PRETEXT system includes other criteria such as
extrahepatic abdominal disease, tumour rupture, metastases, portal and hepatic vein involvement. A revised PRETEXT system in 2005 clarified the criteria but essentially the risk stratification remained the same (Roebuck et al., 2007).

Table 1.2. Definitions of PRETEXT number

<table>
<thead>
<tr>
<th>PRETEXT number</th>
<th>Definition</th>
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<tbody>
<tr>
<td>I</td>
<td>One section is involved and three adjoining sections are free</td>
</tr>
<tr>
<td>II</td>
<td>One or two sections are involved, but two adjoining sections are free</td>
</tr>
<tr>
<td>III</td>
<td>Two or three sections are involved, and no two adjoining sections are free</td>
</tr>
<tr>
<td>IV</td>
<td>All four sections are involved</td>
</tr>
</tbody>
</table>

1.4.1.3 SIOPEL-2

Based on these findings, patients in SIOPEL-2 were treated according to PRETEXT classification. Standard risk (SR) HB patients, i.e. those in whom the tumour has not spread outside the liver and involves up to three liver sections, were treated with Cisplatin alone (Perilongo et al., 2004). The rationale for this was to avoid the risk of cardiomyopathy that may occur many years after treatment with Doxorubicin. Those High Risk (HR) patients, whose tumour involved the whole of the liver or had spread beyond the liver, were treated with more intensive chemotherapy using three drugs, in an effort to improve the prognosis by making the tumour more resectable. Patients with HCC, who have a poor prognosis, were treated with the HR protocol (Cacciavillano et al., 2004).

The results of SIOPEL-2 suggested that Cisplatin alone is effective chemotherapy for SR children. The purpose of the randomized study was to prove that Cisplatin alone is less toxic but as effective as PLADO. Comparison of HR and SR groups showed that the risk stratifications were justified, as the three-year OS for the two groups was 52% and 91% respectively (Perilongo et al., 2004).

1.4.1.4 SIOPEL-3 clinical trial

SIOPEL-3 is the third clinical trial and the main aim was to determine whether single
agent Cisplatin is less toxic and as effective, in terms of response to chemotherapy and resection rate of the tumour, as Cisplatin plus Doxorubicin in SR patients. For HR patients, SIOPEL-3 aimed to evaluate the effect of three drugs, Carboplatin, Doxorubicin and Cisplatin (SuperPLADO), compared with HR patients treated with Cisplatin and Doxorubicin in the SIOPEL-1 trial. The SIOPEL-3 trial closed in 2008 and findings from the trial have shown that Cisplatin monotherapy achieves similar rates of complete resection and survival among children with standard-risk HB as compared with Cisplatin plus doxorubicin. Children treated with Cisplatin alone also had significantly less hematologic toxicity (Perilongo et al., 2009). In the HR arm of the SIOPEL-3 clinical trial, patients were treated with alternating cycles of Cisplatin and Carboplatin plus Doxorubicin prior to surgery. Results from the HR trial showed an improved rate of resection with this treatment and also an improved survival for patients with HR hepatoblastoma (Zsiros et al.).

1.4.1.5 SIOPEL-4 and beyond
SIOPEL-4 looks at HR-HB and HCC patients and aims to evaluate whether more intensive chemotherapy using three drugs, Carboplatin and Doxorubicin alternating with Cisplatin, improves the response rate to chemotherapy and subsequent resection rate compared to SIOPEL-1. SIOPEL-5 and 6 trials are currently open and involve the management of non-cirrhotic HCC and low-risk HB respectively. Future clinical trials will aim to refine the treatment strategy for these tumours by stratification of patients into specific prognostic risk groups. In this study, I aim to identify novel biomarkers that may aid in prognostication and allow for more targeted therapy.

1.4.1.6 Risk Stratification of HB for current SIOPEL studies
The first SIOPEL trial identified PRETEXT staging and lung metastases as the two most important prognostic factors for HB risk stratification (Aronson et al., 2005, Brown et al., 2000). A low serum AFP at diagnosis (<100ng/ml) has also emerged as a risk factor that is associated with poor outcome (De Ioris et al., 2008). Based on this SIOPEL stratifies patients into high-risk (HR) or standard-risk (SR) and treatment is administered accordingly as shown in Table 1.3.
Table 1.3. Risk stratification criteria for SIOPEL

<table>
<thead>
<tr>
<th>High Risk</th>
<th>Standard Risk</th>
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<tbody>
<tr>
<td>Patients with any of the following:</td>
<td>Serum AFP &lt;100ng/ml</td>
</tr>
<tr>
<td></td>
<td>All other patients</td>
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</table>

PreTEXT IV Additional PreTEXT criteria:
- Extrahepatic abdominal disease
- Tumour rupture
- Metastases or lymph node metastases
- Portal vein involvement
- Hepatic vein involvement

1.4.2 Children’s Oncology Group (COG)

Historically, different staging systems have been used for HB in different parts of the world. The Children’s Oncology Group (COG) is involved in most North American clinical trials and this involves postsurgical extent of the disease criterion to stage hepatoblastoma except in cases where there is metastases at diagnosis as described in Table 1.4. In COG trials, the standard approach has been to attempt initial complete resection before initiating chemotherapy. If the tumour cannot be completely resected, the child is started on chemotherapy to decrease the tumour bulk and make it resectable. In children in whom the tumour remains unresectable, alternative therapies such as cisplatin, vincristine, and 5-fluorouracil are used (Reynolds et al., 1992, Katzenstein et al., 2002).

Table 1.4: Traditional COG staging system, with stage and resection margins (Meyers et al., 2009).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Resection</th>
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<tbody>
<tr>
<td>Stage IV</td>
<td>Distant metastatic disease</td>
</tr>
<tr>
<td>COG stage</td>
<td>Resection</td>
</tr>
<tr>
<td>Stage I</td>
<td>Completely resected</td>
</tr>
<tr>
<td>Stage II</td>
<td>Microscopic residual at margins of resected specimen</td>
</tr>
<tr>
<td>Stage III</td>
<td>Partially resected or unresected specimen confined to liver; tumour spill during surgery; positive lymph nodes</td>
</tr>
</tbody>
</table>
1.4.3 German Society for Paediatric Oncology and Haematology (GPOH)
The German study group uses a similar strategy as the COG system to stage HB. Primary surgery was used in stage I and II with stage III and IV treated with preoperative chemotherapy (Roebuck and Perilongo, 2006, von Schweinitz et al., 1995). However, recent studies by the group have recommended preoperative chemotherapy for all HB patients (Fuchs et al., 2002).

1.5 Clinical outcomes for hepatoblastoma
In the last 30 years overall survival from hepatoblastoma has risen from 30% to 70% with improvements in treatment (Schnater et al., 2003). Complete cure of HB is possible only when surgical excision of the tumour is complete. Prior to the use of preoperative chemotherapy, 50-60% of newly diagnosed hepatoblastomas were considered resectable; however complete resection did not occur in all cases (Lack et al., 1982, Tiao et al., 2005). As a result of this and as approximately 20% of HB patients present with metastases, preoperative chemotherapy is becoming more widely used (Fuchs et al., 2002, Roebuck and Perilongo, 2006). Platinum based treatment is now the “gold standard” for HB but it is associated with ototoxicity and nephrotoxicity (Morland, 2006). Recent success with orthotopic liver transplantation in children with unresectable tumours after chemotherapy, has also improved the outcome for HB (D'Antiga et al., 2007, Browne et al., 2008).

Similar overall survival rates of approximately 75% has been found in both the SIOPEL and COG study groups (Roebuck and Perilongo, 2006). In the COG trials, survival rates greater than 90% are seen in children with resectable tumours with the addition of postoperative chemotherapy and a similar survival rate was found by SIOPEL for low risk tumours (Meyers et al., 2009, Morland, 2006). High-risk patients from either study group have around 35% survival rate, while outcomes for children in whom the tumour recurs or progresses while on treatment are poor, with a two-year survival rate of less than 20% (Meyers et al., 2009).

A recent comparison of SIOPEL and COG staging systems by Meyer et al, has shown
that the two staging systems along with SCUD histology and a low serum AFP at diagnosis are important predictors of outcome. The study also acknowledged that worldwide co-operation is vital in developing a common risk stratification for HB that will aid in prognostication and treatment in this rare tumour (Meyers et al., 2009). Future studies should aim to reduce late effects due to chemotherapy and to identify patients who do not respond to current treatment.

1.6 Molecular Pathways involved in HB

1.6.1 Wnt/β-catenin pathway

The aetiology of HB is unknown although a significant tumour subset harbours aberrations in various components of the Wnt/β-catenin pathway that plays an important role in liver development, regeneration and cancer. The disruption of Wnt signalling is seen in many cases of FAP due to mutations in the Adenomatous Polyposis Coli (APC) gene leading to aberrant β-catenin activation. Many children with FAP develop hepatoblastoma and as a result of this link, Wnt/β-catenin was first proposed as a candidate pathway in HB pathogenesis.

The canonical Wnt pathway involves proteins that are directly involved with both gene expression and cell adhesion, with β-catenin the critical mediator of the pathway. Wnts are a highly conserved family of proteins that evolved more than 600 million years ago (Teo et al., 2006). There are nineteen Wnt proteins found in mammals with a conserved primary sequence containing twenty-one specifically spaced cysteine residues (Cadigan and Nusse, 1997a). Genetic analysis of many systems has shown that crucial developmental decisions are controlled by Wnt signalling including cell polarity in Drosophila, axis induction in Xenopus, stem cell proliferation in Cnidarians and gastrulation and organogenesis (Teo et al., 2006, Ai et al., 2006).

Wnt can act through various pathways but it is the stabilization and signalling through β-catenin that is most important in development (Wodarz and Nusse, 1998). The role of β-catenin was initially thought to be solely involved in cell-cell adhesion until the gene was cloned by McCrea et al in 1991 (McCrea et al., 1991). It was found to be
homologous to the gene for Armadillo in *Drosophila* that is involved in wingless pathway signalling and thus its role as a cell signalling molecule was established. Recent studies have shown that Wnt/β-catenin regulates almost all human adult stem cell systems including blood, skin, brain and gastro-intestinal tract (Reya and Clevers, 2005, Radtke and Clevers, 2005). However studies show that Wnt/β-catenin signalling is not necessary for embryonic stem cell regulation (Sato et al., 2004). Wnt signalling has the capacity to induce transient proliferation in adult resting cells, for example intestinal crypt cells (Willert and Jones, 2006) and Wnt regulation of epithelial cell proliferation in the colon exactly balances cell death (Peifer and Polakis, 2000). As there are a wide range of Wnt target genes, signalling through this pathway can have very different outcomes such as proliferation or terminal differentiation (Peifer and Polakis, 2000).

In the absence of Wnt ligands, a degradation complex consisting of APC, Axin and Glycogen synthase kinase 3-β (GSK3β) sequesters monomeric β-catenin (Polakis, 1999). Axin is thought to act as a scaffold by binding to armadillo repeats of β-catenin, GSK3β and the tumour suppressor APC (Price, 2006). Within the complex, GSK3β phosphorylates β-catenin at specific serine and threonine residues as shown in Figure 1.1A. This phosphorylation tags the protein for ubiquitination and subsequent proteasomal degradation (Aberle et al., 1997). Therefore levels of β-catenin are regulated by its degradation.

Figure 1.1B shows Wnt binding to its receptors, Frizzled (Fz) and Low-Density-Lipoprotein receptor related protein (LRP), forming a trimeric complex that targets Dishevelled (Dvl), which is thought to act as a docking site for Axin (Gao and Chen). This results in the phosphorylation of LRP6 by GSK3β and Casein Kinase (CKI), leading to inactivation of the APC/Axin/GSK3β degradation complex (Zeng et al., 2005). In this situation β-catenin does not undergo serine/threonine phosphorylation and is not targeted for proteasomal degradation as illustrated in Figure 1.1C. This results in cytoplasmic accumulation of β-catenin that binds to the TCF/Lef family of transcription factors and can translocate to the nucleus to transactivate expression of target genes. These genes include those coding for c-Myc and Cyclin D1 involved in proliferation, survivin that is involved in antiapoptosis, invasion genes such as matrix
metalloproteinases and VEGF, an angiogenesis factor (Polakis, 1999). Therefore, perturbations in Wnt signalling result in the accumulation of nuclear β-catenin and activation of its target genes.

1.6.2 Role of β-catenin in cell adhesion
β-Catenin also plays a role in cellular adhesion by its binding to Type I cadherins and aids in cell structural organization by linking E-cadherin with the axin cytoskeleton (Jamora and Fuchs, 2002). There appears to be some cross talk between adhesion and signalling pathways due to the involvement of β-catenin. In a mouse model it has been shown that E-cadherin down-regulated β-catenin/TCF mediated transcriptional activity by sequestering β-catenin from the cytoplasmic pool into E-cadherin cell-adhesion complexes even in the presence of Wnt signalling (Herzig et al., 2007). It has been proposed that a decrease in E-cadherin at the cell membrane leads to the accumulation of free β-catenin available for TCF/Lef mediated transcription (Huber et al., 1996). Recent studies have shown that phosphorylation of tyrosine residue 142 of β-catenin by tyrosine receptor kinases (TRKs) such as c-Met, causes dissociation of the cadherin-catenin complex by blocking the association with α-catenin, and promotes binding of β-catenin to the nuclear co-factor Bcl9-2 (Brembeck et al., 2004, Piedra et al., 2003). Phosphorylation of β-catenin at tyrosine residue 654 by activated c-Met causes dissociation of the β-catenin/Met complex at the cell membrane and also blocks binding to E-cadherin (Brembeck et al., 2006, Roura et al., 1999).
Figure 1.1: Model for activation of the Wnt/β-catenin pathway. A In the absence of Wnt signaling, β-catenin is phosphorylated and targeted for proteasomal degradation by a destruction complex that contains Axin, APC and Gsk3β. B On binding of Wnt to its receptors Fz and LRP, Dvl binds to Fz and recruits the destruction complex through interactions with Axin. Gsk3β and CK phosphorylate LRP, which then acts as a docking site for Axin. C Binding of Axin to LRP leads to inhibition of the destruction complex and stabilization of β-catenin (Adapted from Fuerer, Nusse2007).
1.6.3 Wnt/β-catenin in Liver

The role of the Wnt/β-catenin pathway in liver has been intensively studied. Studies in mice have shown that β-catenin is critical for early embryonic liver development with high levels coinciding with a high level of hepatocyte proliferation (Apte et al., 2007). Tan et al also showed that β-catenin is necessary for hepatocyte maturation and expansion in the embryonic liver and is essential for survival (Tan et al., 2008). In the post-natal phase, an increase in β-catenin is seen that coincides with liver growth and as the liver develops the nuclear/cytoplasmic ratio of β-catenin decreases (Gonzalez, 2006). Liver-specific knock-out of β-catenin in mice results in small livers and, conversely, transgenic mice with over-expression of wild-type (WT) β-catenin have livers that are around 15% bigger than normal (Apte et al., 2007, Nejak-Bowen and Monga, 2008). Hussain et al have proposed that β-catenin signalling may be responsible for differentiation into the two major cell lineages in the liver, hepatocytes and biliary epithelial cells (Hussain et al., 2004).

Wnt/β-catenin signalling appears to be quiescent in the normal adult liver. A mouse model with liver-specific knockout of β-catenin showed no major impact in the mature liver (Sekine et al., 2006). However, β-catenin regulates expression of genes involved in ammonia and xenobiotic metabolism in the adult liver (Nejak-Bowen and Monga, 2008) and Wnt/β-catenin plays a vital role in liver regeneration following injury or hepatectomy (Monga et al., 2001).

1.6.4 The role of Wnt/β-catenin signalling in Cancer

Wnt signalling regulates cell proliferation and differentiation in adult tissues such as the epithelial lining of the gut where colonic cells proliferate at a rate that balances cell death (de Lau et al., 2007). When needed, cells receive proliferation signals and when sufficient cell numbers are reached the proliferation stops. Cancer results, in part, from mutations that cause continuous proliferation signals to be sent to cells. Such mutations can cause proteins to be aberrantly expressed (oncogenes) or inactivated (tumour suppressor genes). Several components of the Wnt signalling pathway have been implicated in human tumours or experimental cancer models. Wnt was first identified as an oncogene in mouse mammary tumours (Nusse and Varmus, 1982). Separate studies that identified mutations in the APC gene that interacts with
β-catenin in FAP syndrome provided the connection between Wnt signalling and cancer and aberrant β-catenin accumulation is a common finding in many cancers (Rubinfeld et al., 1993, Su et al., 1993). Upregulation of β-catenin expression in cancer can be caused in many ways: mutations in APC, mutations in Axin, an increase in the Wnt receptor Fz, inactivation of GSK3β by Akt or by mutations or deletions in CTNNB1. Figure 1.2A shows the structure of the β-catenin protein and Figure 1.2B details the mutations found in the GSK3β binding region of the protein seen in cancers.

1.6.4.1 Wnt/β-catenin in Colorectal Cancer

Wnt/β-catenin signalling was first found to be important in colorectal cancer through the study of patients with FAP who have a germline mutation in the APC gene (Rubinfeld et al., 1993). It was found that β-catenin binding by the degradation complex was reduced which allowed accumulation of the protein. Transfection with WT APC led to a decrease in the activity of β-catenin (Munemitsu et al., 1995). Almost 100% of FAP patients carry a germline mutation in APC and >65% of sporadic colorectal cancers also have acquired mutations in APC that block formation of the β-catenin binding complex. Mutations in CTNNB1 have been found in around 50% of colorectal cancers lacking APC mutations and they appear to be mutually exclusive (Morin et al., 1997, Sparks et al., 1998). Mutations in the Axin gene preventing β-catenin targeting by the degradation complex have also been found in colorectal cancers (Segditsas and Tomlinson, 2006).

1.5.4.2 Wnt/β-catenin in Melanoma

Melanoma was one of the first types of tumour to be examined for aberrant Wnt/β-catenin pathway activation. Overexpression of β-catenin has been reported in melanoma cell lines and tumours, and studies by Rubinfeld et al and Rimm et al both showed high levels of protein expression (Rimm et al., 1999, Rubinfeld et al., 1997). But while the former found this expression was associated with CTNNB1 mutations in the GSK3β-binding region, the study by Rimm et al (1999) found that β-catenin mutations were very rare in their melanoma cohort. Overall β-catenin and APC mutations have each been found in 5% of melanomas.
Figure 1.2. β-catenin protein and mutations seen in cancer. A. Schematic diagram of β-catenin protein showing the armadillo repeats (blue boxes) and the N-terminal consensus GSK3β phosphorylation domain (GSK). The two transcriptional domains are indicated by Tx. B. Amino acid sequence of the regulatory region of wild-type (WT) β-catenin and reported changes in various cancers. The residues of the ubiquitination motif are underlined and the putative GSK3β serine phosphorylation residues are indicated in bold. Residues in red indicate mutations found in our study. Endom, endometrial cancer. MB, medulloblastoma. (Morin, 1999).
1.6.4.3 Wnt/β-catenin in Endometrial and Ovarian Cancer

A study by Fukachi et al. found that 13% of their cohort of endometrial cancers had mutations in the serine/threonine phosphorylation region of CTNNB1 and reported β-catenin accumulation in 38% of tumours (Fukuchi et al., 1998). No APC mutations were detected. However, Mirabelli-Primdahl et al. found a much higher rate of mutation (45%) in endometrial cancers (Mirabelli-Primdahl et al., 1999). Interestingly, several studies of ovarian cancers showed that only the endometrioid type harboured CTNNB1 mutations and β-catenin accumulation, and this type of ovarian cancer is histologically similar to colon and endometrial cancers (Palacios and Gamallo, 1998, Wright et al., 1999). CTNNB1 mutation has also been linked with a favourable prognosis in endometrioid ovarian cancer (Gamallo et al., 1999, Palacios and Gamallo, 1998). Mutations in Axin and APC have also been reported in low frequency in this tumour type (Wu et al., 2001).

1.6.4.4 Wnt/β-catenin in Gastrointestinal Carcinoid Tumours

Two separate studies have demonstrated high levels of β-catenin accumulation in gastrointestinal carcinoid tumours, which are neuroendocrine tumours of the gastrointestinal tract (Fujimori et al., 2001, Su et al., 2006). The study by Fujimori et al. found point mutations in the CTNNB1 gene predominantly at serine 37, in 37.5% of tumours and one tumour with an APC mutation (Fujimori et al., 2001). In contrast, the larger study by Su et al. did not find any associated mutations in CTNNB1 or APC (Su et al., 2006).

1.6.4.5 Wnt/β-catenin in Medulloblastoma

β-Catenin protein accumulation and gene mutation is a common event in the pathogenesis of medulloblastoma, the most common brain tumour in children. Interestingly, mutations appear to be exclusively found in tumours that have nuclear expression of β-catenin (Ellison et al., 2005, Fattet et al., 2009). Both of these studies have linked nuclear accumulation of β-catenin with a good prognosis in medulloblastoma. Mutations in Axin and APC, that may cause activation of β-catenin, have also been reported in medulloblastoma (Eberhart et al., 2000, Dahmen et al., 2001).
1.6.4.6 Wnt/β-catenin in Hepatocellular Carcinoma (HCC)

Aberrant accumulation of β-catenin has been reported in 20% - 40% of hepatocellular carcinomas. Mutations and deletions affecting the GSK3β phosphorylation region in exon 3 of β-catenin are also common events in HCC and hepatoma cell lines. The Catalogue of Mutations in Cancer (COSMIC) database (www.sanger.ac.uk) has reported mutations in 17% of almost 3000 HCC tumours from numerous studies. Of these 65% are missense mutations and 18% are deletions in exon 3. Similar to medulloblastoma, Wong et al found that mutations were predominantly associated with nuclear β-catenin expression and nuclear expression correlated with a better outcome than cytoplasmic accumulation of the protein (Wong et al., 2001). However, no correlation has been found between nuclear β-catenin accumulation and expression of its transcription targets (Inagawa et al., 2002, Prange et al., 2003). Earlier studies found an association between β-catenin expression and cell proliferation, but this was not corroborated in a larger study by Prange et al (Nhieu et al., 1999, Prange et al., 2003). Zucman-Rossi et al looked at both β-catenin mutations and Axin mutations in HCC and concluded that loss of function of Axin does not give a gain of function of β-catenin due to different levels of transcription targets seen (Zucman-Rossi et al., 2007). Mutations in Axin have been reported in 5-14% of HCC and an upregulation of Fz has also been implicated in β-catenin activation in HCC (Merle et al., 2004, Nhieu et al., 1999, Taniguchi et al., 2002, Satoh et al., 2000). A recent study by Cieply et al has found that mutations in the β-catenin gene are associated with an aggressive phenotype in HCC (Cieply et al., 2009).

1.6.4.7 Wnt/β-catenin in Hepatoblastoma

The role of β-catenin in HB was first investigated by Koch et al (1999). They found that 48% of samples tested had mutations or deletions in exon 3 of CTNNB1. Since then several studies have uncovered similar results with mutation frequencies of up to 90% being reported and deletions accounting for up to 50% of these mutations as reviewed in Table 1.5 (Blaker et al., 1999, Jeng et al., 2000, Koch et al., 2005, Takayasu et al., 2001, Taniguchi et al., 2002, Udatsu et al., 2001, Wei et al., 2000, Yamaoka et al., 2006).
In contrast, studies by Park et al and Curia et al found a much lower frequency of mutation in HB (Curia et al., 2008, Park et al., 2001b). All studies investigating expression of β-catenin protein by immunohistochemistry (IHC) found it to be aberrantly expressed in up to 100% of cases (Jeng et al., 2000, Takayasu et al., 2001, Wei et al., 2000, Yamaoka et al., 2006). The majority of studies found no correlation between β-catenin mutation or overexpression and histologic type or disease outcome. One exception to this is the study by Park et al that found nuclear accumulation of β-catenin correlated with embryonal HB and a poor outcome (Park et al., 2001b). Since CTNNB1 mutations were only found in a portion of HBs with β-catenin accumulation many studies also looked for defects in other components of the Wnt pathway. APC mutations are very rare findings while Axin mutations may account for 7% of aberrant Wnt/β-catenin signalling (Koch et al., 1999, Takayasu et al., 2001, Taniguchi et al., 2002, Udatsu et al., 2001). Interestingly, high levels of the Wnt antagonists Nkd-1, β-TrCP, Axin2 and Dkk-1 were found in a HB study by Koch et al (Koch et al., 2005). These molecules would normally suppress Wnt signalling via a negative feedback loop. This may be abrogated in HB due to other genetic alterations that stabilize β-catenin. A recent study by Lopez-Terrada et al suggested that all HBs might arise from proliferating bipotential precursors due to the expression of Dlk1, a marker of bipotential oval cells (Lopez-Terrada et al., 2009). This study also found that Wnt signalling was associated with embryonal and mixed HB while Notch signalling was more predominant in fetal HB.

### 1.5.5 HGF/c-Met Signalling Pathway

Another signalling pathway thought to be active in hepatoblastoma development is the Hepatocyte growth factor (HGF)/c-Met pathway. HGF or Scatter factor (SF) was identified separately as a potent growth stimulus for primary hepatocytes in culture (Nakamura et al., 1986) and a molecule that induces epithelial cell scatter; the process by which epithelial cells dissociate and move away from one another (Stoker et al., 1987). It was later proved to be the same molecule (Naldini et al., 1991). HGF is secreted by mesenchymal cells e.g. myofibroblasts, and plays an important role in proliferation, motility and morphogenesis (Maulik et al., 2002b). These activities are crucial during the processes of embryogenesis, wound healing, tissue regeneration, angiogenesis, growth and morphologic differentiation. HGF has been shown to be
vital for prenatal liver development and the most important factor for liver regeneration after hepatectomy (Michalopoulos and De Frances, 1997, Schmidt et al., 1995). The proto-oncogene product of c-Met was identified as the receptor for HGF by Bottaro et al and is normally expressed by most epithelial and endothelial cells (Bottaro et al., 1991). Met is a heterodimeric receptor tyrosine kinase. Upon ligand binding, Met becomes phosphorylated at tyrosine residues in its receptor tail and activates many downstream signalling pathways for cellular proliferation (e.g. MAP kinase) and cell motility (e.g. PI3K) (Royal et al., 1997, Ponzetto et al., 1996). Schmidt et al demonstrated the importance of Met in embryogenesis whereby mice die in utero due to null mutations (Schmidt et al., 1997).

1.6.5.1 The role of HGF/c-Met signalling in cancer

“Invasive growth” is a biological program that allows tumour cells to invade adjacent tissue and to proliferate and survive in distant sites (Benvenuti and Comoglio, 2007). This tumorigenic process uses many mechanisms also seen in normal organogenesis, organ regeneration and wound healing including those activated by the HGF/c-Met pathway. The metastatic potential of HGF/c-Met was first demonstrated in cell lines by Rong et al (Rong et al., 1994) and it has been shown since that mutations in c-met, over-expression of c-Met or increased activation of the ligand/receptor loop can all contribute to tumorigenesis. Mutations of the c-Met gene are rare occurrences that constitutively activate the tyrosine kinase activity and have been reported in renal cell carcinomas, childhood HCC, gastric cancers and gliomas (Maulik et al., 2002b). Over-expression of c-Met is a more common finding and has been reported in many cancers including, but not limited to, breast, lung, prostate, endometrial, colorectal and liver cancer (Fujita and Sugano, 1997, Jin et al., 1997, Kurimoto et al., 1998, Maulik et al., 2002a, Suzuki et al., 1994, Wagatsuma et al., 1998). Over-expression of c-Met can be caused by increased copy number of the gene or by control at the transcriptional level. The co-expression of HGF and its receptor c-Met is also commonly seen and has been implicated in the increased invasion and metastatic potential of cell lines and higher tumour grade and poor prognosis in tumours such as liver and breast (D'Errico et al., 1996, Ghoussoub et al., 1998).
1.6.5.2 HGF/c-Met and hepatoblastoma

The role of HGF in hepatoblastoma has been recognized as early as 1998 when it was seen that elevated levels of the growth factor were present in the serum of a large proportion of children following surgery (von Schweinitz et al., 1998). This and a further study by the same group found that there was a very rapid development of recurrent tumour when the excision was incomplete and that the residual tumours exhibited high levels of c-Met. They postulated that liver regeneration occurring after hepatectomy causes growth of the residual tumour due to activity of HGF.

Proliferation of HB cell lines in response to HGF indicates that this may be the case (von Schweinitz et al., 2000) although a more recent study shows that HGF/c-Met signalling does not lead to proliferation of HB but rather it protects against chemotherapy-induced apoptosis (Grotegut et al.).

1.6.5.3 HGF/c-Met signalling through β-catenin activation

It was first recognized by Papkoff et al that HGF signalling leads to a transient decrease in GSK3β activity and subsequent nuclear accumulation of β-catenin, independent of Wnt signalling (Papkoff and Aikawa, 1998) and around the same time it was shown that scattering of colorectal cell lines in response to HGF was associated with increased tyrosine-phosphorylated β-catenin (Hiscox and Jiang, 1999). Further studies revealed that oncogenic mutations that constitutively turn on tyrosine kinase activity of c-Met, cause activation of β-catenin (Danilkovitch-Miagkova et al., 2001). This work showed that upon ligand binding, c-Met tyrosine-phosphorylates β-catenin, which blocks its interaction with Axin and GSK3β and protects it from ubiquitination and degradation. This leads to accumulation and nuclear translocation of β-catenin and also targets the co-transcription factor, Bcl9-2 as shown in Figure 1.3C. Monga et al demonstrated a novel association of β-catenin with Met at the inner surface of the cell membrane in rat hepatocytes and that this association is lost and β-catenin accumulates in the nucleus in response to HGF (Monga et al., 2002). They also showed that about 80% of Met is associated with β-catenin at the cell membrane and about 30-40% of the total β-catenin is associated with Met and the rest with E-cadherin as shown in Figure 1.3A. A more recent study selectively mutated tyrosine residues in β-catenin and found that phosphorylation of tyrosine residues 654 and 670 are crucial to regulate the interaction with Met (Zeng et al., 2006a). Figure 1.3B
shows that phosphorylation of these and tyrosine residue 142 correlates with disassociation of cadherin complexes, decreased cell adhesion and increased cell mobility. This may allow reorganization of the cells during remodelling of the regenerating liver and may confer properties of invasion and metastasis to tumour cells (Michalopoulos and DeFrances, 1997, Monga et al., 2001).

1.6.5.4 c-Met/β-catenin activation in Cancer
The first report of c-Met/β-catenin activation in cancer came in a study of breast cancer in 2000 (Nakopoulou et al., 2000), which examined Met expression in conjunction with β-catenin expression and found a correlation with favourable prognosis in their cohort. This group also suggested that tyrosine phosphorylation of β-catenin might be occurring. Herynk et al later showed that activation of c-Met in colorectal cell lines leads to constitutively tyrosine phosphorylated β-catenin and higher metastatic potential (Herynk et al., 2003). A further study in colorectal carcinoma has identified a functional feedback loop between Met and β-catenin (Rasola et al., 2007). They demonstrated increased transcription of Met in response to Wnt stimulation in early dysplasia, and this has been shown to amplify biologic processes crucial for sustaining colorectal cancer cell invasion and growth. Similarly, Tward et al found that phosphorylated Met and β-catenin activity was seen in HCC but not in hepatic adenomas in a mouse model (Tward et al., 2007). A small IHC study of thirteen hepatoblastomas demonstrated increased expression of both Met and β-catenin in all biopsies with a marked decrease in Met staining post-chemotherapy (Ranganathan et al., 2005a). Grotegut et al found that HGF signalling had no impact on cell proliferation in HB but it did confer anti-apoptotic properties and contributes to chemotherapeutic resistance in HB (Grotegut et al.).
Figure 1.3: Model for activation of the HGF/c-Met/β-catenin pathway. A In the quiescent cell, β-catenin is sequestered at the cell membrane by E-cadherin and Met. B On binding of HGF to its receptor c-Met, c-Met tyrosine phosphorylates β-catenin at residues 142 and 654. C Phosphorylation of tyrosine 142 blocks binding with E-cadherin and promotes binding with the co-transcription factor Bcl9-2. Phosphorylation of tyrosine 654 causes dissociation from c-Met and blocks degradation of β-catenin by the GSK3β destruction complex.
1.7 Epithelial-to-Mesenchymal transition (EMT)
Greenburg and Hey first described EMT in the 1980’s as a reversible process involved in early embryonic development (Greenburg and Hay, 1982). EMT is essential for numerous developmental processes including gastrulation, mesoderm formation and neural tube formation (Kelleher et al., 2006). EMT is a program of development of biological cells characterized by loss of cell adhesion, loss of cell polarity, and increased cell mobility as epithelial cells take on characteristics of mesenchymal cells as shown in Figure 1.4 (Sabbah et al., 2008). EMT is marked by a decrease in E-cadherin, cytokeratin and desmoplakin and a concurrent increase in the abundance of N-cadherin, vimentin, fibronectin and metalloproteinases. Nuclear accumulation of β-catenin, Slug, Snail and Twist are also associated with EMT. There are three different types of EMT; Type 1 is seen in embryogenesis and tissue remodelling, Type 2 is found in wound healing and regeneration and Type 3 is involved in neoplasia. Type 1 and 3 are the most similar although common elements underlie all types (Choi and Diehl, 2009). Studies in EMT are carried out mainly in cell culture and therefore may not entirely reflect the process in vivo. As depicted in Figure 1.4, EMT is believed to be a dynamic series of events that occur sequentially in response to different signals, beginning with a loss of cell-cell contact and decreased polarity leading to a motile phenotype (Hay, 1995).

1.7.1 EMT in the liver
Studies of cultured liver cells have shown that both hepatocytes and cholangiocytes are capable of EMT (Choi and Diehl, 2009). Activation of rat hepatocytes by TGFβ signalling leads to a reduction in epithelial markers and a concurrent increase in mesenchymal gene expression and cell migration (Zeisberg and Neilson, 2009). Studies in liver regeneration indicate that EMT occurs during that process. EMT is not involved in cell lineage specificity and therefore only reflects the plasticity of the cells (Choi and Diehl, 2009). Studies of EMT in hepatocellular carcinoma have indicated a role for EMT (van Zijl et al., 2009). They found increased involvement of Wnt/β-catenin and TGFβ signalling was crucial to metastasis in HCC. A separate study by Zhai et al showed loss of E-cadherin and accumulation of nuclear β-catenin was associated with HCC (Zhai et al., 2008).
1.7.2 The potential role of EMT in cancer development

There is mounting evidence that epithelial to mesenchymal transition contributes to metastasis of tumour cells (Thiery, 2002). Initiation of metastasis involves invasion, which has many phenotypic similarities to EMT, including a loss of cell-cell adhesion mediated by E-cadherin repression and an increase in cell mobility and this EMT signature is seen at the invasive front in many cancers (Weinberg, 2008b). Several oncogenic pathways (TGF-β, peptide growth factors, Src, Ras, Wnt/beta-catenin and Notch) induce EMT (Garber, 2008, Weinberg, 2008a) and most of these signalling pathways converge at the induction of the E-cadherin repressors, and in particular, of the Zeb and Snail genes (Iwatsuki et al.). For example β-catenin transcriptionally activates Slug/Snail, which is then able to bind the E-cadherin promoter. This repression of E-cadherin leads to loss of cell-cell adhesion and increased free β-catenin, which serves to amplify and sustain Wnt signalling (Nelson and Nusse, 2004). HGF has recently been shown to contribute to epithelial cell scattering through MAP kinase upregulation of Snail and repression of E-cadherin (Grotegut et al., 2006). E-cadherin acts as a tumour suppressor in cell lines and a decrease of the
protein caused invasion in a mouse model (Semb and Christofori, 1998). The characteristic finding of decreased expression of E-cadherin in EMT has also been linked with progression, metastasis and poor prognosis in a variety of cancers (Thiery et al., 2009). Due to the cross-talk between the adhesion and signalling pathways it is possible that a decrease in E-cadherin leads to accumulation of free β-catenin with an increase in associated transcription (Huber et al., 1996). Conversely, it has been proposed that any disruption of the intracellular cadherin-catenin complex e.g. due to β-catenin mutations that block E-cadherin binding, would lead to loss of cell adhesion and consequent metastasis (Christofori and Semb, 1999). Herzig et al have shown that loss of E-cadherin is the rate-limiting step in the progression from adenoma to carcinoma in a mouse model (Herzig et al., 2007) but that loss of E-cadherin or activation of β-catenin alone are not sufficient for tumour progression in mice. It is more likely that a complex mechanism such as EMT involving various pathways such as Wnt/β-catenin and HGF/c-Met contributes to tumour progression.

1.8 MicroRNAs
MicroRNAs (miRNAs) are a class of small endogenous non-coding RNA molecules. They are generally around 21-24 nucleotides in length and were first discovered in the nematode (Lee et al., 1993). Since then thousands of miRNAs have been identified in a variety of species. The most current release from the miRBASE database estimates in excess of 10,000 miRNA sequences including more than 700 isolated from humans (http://microrna.sanger.ac.uk). The number of miRNAs discovered is rising rapidly and to avoid confusion an annotation system has been suggested to designate individual miRNAs e.g. hsa-miR-121, with “hsa” standing for homo sapiens, miR for microRNA and the number the order of discovery (Li et al.). Computational algorithms have been developed including miRANDA, TargetScan and mirBASE that identify putative targets and they estimate that there may be thousands of human miRNAs that regulate in excess of one third of coding genes but this has yet to be proven (Wiemer, 2007, Liu et al., Thomson et al.).

All known and predicted protein-coding genes only account for ~2% of the genome and most miRNAs are found in regions of the genome that were thought to be non-coding although some miRNAs have been found in introns and exons of protein-
coding genes. MiRNAs are commonly, but not always, found in clusters sharing the same promoter and can sometimes be co-transcribed with their target genes e.g. hsa-miR-483 located within intron 2 of the \textit{IGF2} locus (Veronese et al.). miRNAs regulate gene expression at the post-transcriptional level by base-pairing, usually with partial complementarity in the 3’UTR, to their target mRNAs. This results in translational repression or degradation via the RNAi pathway (Bartel, 2004).

The region of the miRNA that controls its target specificity is usually only six nucleotides in length and called the “seed sequence” (Lewis et al., 2005). Based on seed-sequence matches, each miRNA is predicted to target tens to hundreds of genes and conversely many genes have target sites for multiple miRNAs (Grimson et al., 2007, Krek et al., 2005). This may allow for faster and more efficient synchronization of regulatory effects, referred to as “translation on demand” (Beyer et al., 2004). In addition to target specificity, miRNAs also show tissue-specific and developmental stage-specific patterns of expression, e.g. hsa-miR-1 is heart and muscle specific and hsa-miR-21 found in fetal but not adult liver (Girard et al., 2008, Zhao et al., 2007). It has been shown that miRNAs are implicated in almost every cellular process including metabolism, differentiation and cell cycle control although only a small fraction of miRNAs have been verified and annotated (Bartel, 2004, Wiemer, 2007).

1.8.1 MicroRNA Biogenesis

MicroRNA genes are transcribed by RNA polymerase II as long primary miRNAs (pri-miRNAs) that contain one or more stem-loop or hairpin sequence (See Figure 1.5). Cleavage of the stem-loop/hairpin by Drosha gives rise to a pre-miRNA of around 70 nucleotides that is transported to the cytoplasm by Exportin-5 (Lee et al., 2003). In the cytoplasm the endonuclease Dicer, along with its ds-RNA binding partner, TRBP, cleaves the pre-miRNA into a 20-25 nucleotide imperfect duplex (Tijsterman and Plasterk, 2004). TRBP recruits the argonaute protein and together these comprise the RNA-induced silencing complex (RISC). The passenger strand is removed before the miRNA targets the RISC complex to the mRNA. Bartel \textit{et al} found that several miRNAs, either the same or different, must bind to a target for translational repression to occur (Bartel, 2004).
Figure 1.5: Biogenesis of miRNAs. Genes encoding miRNAs are transcribed into primary transcripts (Pri-miRNAs) then processed into precursor miRNAs (Pre-miRNAs) by the nuclear RNAse III enzyme, Drosha. The pre-miRNAs are transported to the cytoplasm by Transportin-5 and further processed into mature miRNAs by the ribonuclease, Dicer and incorporated into the RNA-induced silencing complex (RISC). Mature miRNAs interfere with mRNA translation by degradation or translational repression (Chen, 2009).

1.8.2 The role of MicroRNAs in cancer
The first study linking microRNAs with cancer was the identification of a non-protein coding region deleted in chronic lymphoblastic leukaemia (CLL) patients (Calin et al., 2002). Since then the role of miRNAs in tumorigenesis has been widely studied in various cancers. Expression profiling using micro-array technology has identified miRNA patterns associated with different tumour types (Volinia et al., 2006) and the current hurdle is to define roles for the individual miRNAs associated with cancer. The majority of miRNAs are believed to act as tumour suppressors and are down-regulated as the tumour becomes less differentiated (Gregory et al., 2008). However certain miRNAs are up-regulated in cancer and those with proven oncogenic properties are termed “oncomirs” (Esquela-Kerscher and Slack, 2006). miRNAs with
tumour suppressor function include members of the let-7, miR-34 and miR-15 families and proven oncomirs include miR-21 and the miR-17-92 family (Medina and Slack, 2008). Testing of putative miRNA targets has shown that common cancer pathway molecules e.g. BCL2, MYC, p53 and PTEN are regulated by or regulate expression of miRNAs during cancer progression (Lotterman et al., 2008). Mir-21 has recently been implicated in chemoresistance in glioblastoma cells while miR-199a-3p influences doxorubicin sensitivity in HCC cells by regulation of mTOR and c-Met (Fornari et al., Shi et al.). Hypermethylation of miRNA genes has been proposed as one way in which their expression is controlled in cancer metastasis (Lujambio et al., 2008) and specific inactivation of miR-34a by CpG methylation is seen in many cancers (Lodygin 2008).

1.8.3 miRNAs in Liver

Comparative studies of different human tissues have identified organ specific miRNA profiles (Liang et al., 2007). miR-122 appears to be specific for liver (Chang et al., 2004) and many others including miR-1, miR-16, miR-143 and the let-7 family are also abundant in adult liver (Meng et al., 2007b, Girard et al., 2008). Interestingly, fetal liver shows a different profile with miRNAs such as miR-21, miR-199a and miR-92a more abundant (Girard et al., 2008). This implies a differential role for miRNAs during embryonic development in the liver. A recent study has reported a possible role for miRNAs during hepatocyte proliferation in liver regeneration after hepanectomym in mice (Song et al.). Several studies have reported aberrant miRNA expression in HCC. The specific role of these miRNAs is largely unknown although it was shown that miR-21 is involved in carcinogenesis by its regulation of the tumour suppressor, PTEN (Meng et al., 2007a) and CyclinG1 is a target of miR-122a, which is frequently down-regulated in HCC (Gramantieri et al., 2007). Ladeiro et al found that miRNA profiles were often associated with clinical features, risk factors and gene alterations in HCC (Ladeiro et al., 2008). The results of a recent study of more than 200 samples found that miR-221 overexpression significantly contributes to liver disease progression from normal through cirrhosis to HCC (Pineau et al.). Studies in HCC cell lines have also suggested a role for miRNA in c-Met activated tumorigenesis (Salvi et al., 2009). Zheng et al investigated the effect of miR-34a on c-Met expression in HCC cell lines and found that transfection with miR-34a inhibits
tumour cell migration and invasion in a c-Met dependant manner (Li et al., 2009). Decreased levels of c-Met were seen in both cell lines and HCC tumours. miR-199a-3p has also been shown to regulate c-Met in HCC cells and may influence doxorubicin sensitivity in HCC (Fornari, 2010). A single small study using array analysis found that hepatoblastoma could be distinguished from adjacent normal tissue and from HCC based on miRNA expression profiles (Magrelli et al., 2009).

1.8.4 Regulation of epithelial-mesenchymal transition by MicroRNAs
Recently, miRNAs have appeared as a powerful regulator of EMT. For example, Twist induces miR-10b transcription, which induces EMT in cancer cells and this has been shown to facilitate cancer metastases (Wu and Zhou, 2008). Expression of miR-200 and miR-205 family induces upregulation of E-cadherin in cancer cell lines, and loss of miR-200 is commonly found in invasive breast cancer cell lines and in metaplastic breast tumours lacking E-cadherin (Park et al., 2008). The miR-200 family works by down-regulating the E-cadherin repressors ZEB1 and ZEB2 (Gregory et al., 2008). miR-155 and mir-29a are thought to regulate EMT through their interaction with TGF-β signalling, a major effector of EMT, in breast cancer (Kong et al., 2008, Gebeshuber et al., 2009). A recent study that looked at global gene and miRNA expression profiling in adult and embryonic liver revealed a negative correlation between let-7c and the receptor for TGFβ that was verified in hepatocellular carcinoma cell lines (Tzur, 2009). Therefore, low levels of let-7c observed in embryonic liver may play a role in maintaining TGFβ signalling, which is thought to promote hepatocyte proliferation and is involved in EMT.
1.9 Thesis hypotheses and aims

The molecular mechanisms underlying hepatoblastoma, and indeed most paediatric cancer, are poorly understood. Elucidation of the molecular pathways involved and how these pathways are controlled will be useful in the clinical setting and also contribute to our knowledge about cancer pathogenesis and normal liver development. It is my hypothesis that:

(i) That specific proteins implicated in HB biology would be differentially expressed in tumours of relapse compared to non-relapse patients and between histologic subtypes.

(ii) That signaling through the HGF/c-Met pathway may account for the high level of β-catenin expression in HB.

(iii) That miRNA profiles may differentiate between tumour subtypes and predict response to therapy in HB and that specific miRNAs may contribute to the pathogenesis of HB.

To test the validity of these hypotheses this thesis aims to:

(i) Examine protein expression of the chosen biomarkers in a large cohort of HB patients using IHC and correlate the results to histologic and clinical parameters (Chapter 3).

(ii) Examine mutation status of the gene for β-catenin and c-Met activation of β-catenin in our cohort and relate the results to the overall expression of β-catenin in the tumours.

(iii) Examine miRNA expression profiles from a selection of our tumour samples using miRNA microarray technology.
Chapter Two

Materials and Methods
Chapter 2: Materials and Methods

2.1 Samples

2.1.1 Hepatoblastoma Patient Cohort

Our cohort comprises HB patients from the SIOPEL 3 clinical trial who were randomized to receive either cisplatin (CDDP) or a combination of cisplatin and doxorubicin (PLADO) preoperatively. Under the auspices of SIOPEL, participating centres were requested to send us formalin-fixed paraffin-embedded (FFPE) hepatoblastoma tumour samples on a voluntary basis at the discretion of the pathologists involved. Table 2.1 details a list of participating centres.

Samples on more than 120 cases were received. Some of these were omitted from the study as the paraffin blocks sent were fine needle biopsies or biopsies with scant tissue. Removal of a core of this tissue would completely use up any remaining sample. Some of the samples were found, on examination of a H+E section, to lack any residual tumour. Several samples sent were not on the SIOPEL-3 trial.

In total 91 HB cases were accessed for this study with clinical, histologic and survival data available for most samples. A single 4µm section was cut from each paraffin block and the slides stained with haematoxylin and eosin (H+E). Each slide was examined locally by a pathologist (Dr Carina Miles), to select representative tumour and adjacent normal tissue for tissue-microarray construction.

2.1.2 Fetal Liver control samples

Fetal liver control tissue was sourced with the help of Dr. Carina Miles from fetal autopsy specimens. FL14 is normal liver from a 14-week old fetus with multiple congenital abnormalities. FL20 comprises normal liver at 20 weeks gestation from a fetus with Ch47XY and Ch18 chromosomal abnormalities. These samples were snap-frozen and stored at -80°C.
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<td>Sweden</td>
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<td>Great Ormond Street Hospital for Children</td>
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<td>England</td>
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<td>Northern Ireland</td>
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<tr>
<td>Royal Victoria hospital</td>
<td>Belfast</td>
<td>Northern Ireland</td>
</tr>
<tr>
<td>Birmingham Children's Hospital</td>
<td>Birmingham</td>
<td>England</td>
</tr>
<tr>
<td>Hospital Materno-Infantil</td>
<td>Malaga</td>
<td>Spain</td>
</tr>
</tbody>
</table>
2.1.3 Adult Liver control tissue
Six samples of non-tumour liver tissue were obtained from Cancer Society Tissue Bank, Christchurch. The samples were from two men and four women ranging in age between 51 and 82 years at the time of surgery (See Table 2.2). The fresh liver tissue was divided and half snap-frozen and half formalin-fixed and paraffin-embedded.

Table 2.2: Adult liver control samples obtained from the Cancer Society Tissue Bank

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gender</th>
<th>Age at surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>76</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>56</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>82</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>61</td>
</tr>
</tbody>
</table>

2.1.4 Mouse liver control samples
Mouse liver for use as controls were kindly donated by the Cardioendocrine Research Group, University of Otago, Christchurch. Three mice were euthanized by halothane anaesthesia followed by removal of livers. The livers were divided and half of each was placed in RNa later® fresh tissue storage solution (Ambion, Austin, TX, USA) and the other half formalin-fixed and paraffin-embedded.

2.2 Ethics
All clinical data and tumour tissue used in this study has been collected with informed consent under ethics approvals from institutional ethics committees of the participating centres. All experimental procedures were carried out with ethical approval of the Multi-region Ethics Committee of the Ministry of Health (approvals CTY/01/10/141 and CTY/01/10/142).

2.3 Acknowledgements of practical assistance within this thesis
Chapter 3: H+E slides prepared by the candidate were examined by Dr. Carina Miles, and areas of tumour and normal tissue were marked. Following construction of the tissue microarray and IHC by the candidate, the IHC slides were assessed and
scored by the candidate with the help of Dr. Carina Miles and Dr. Clinton Turner. Statistical analysis for Chapter 3 was carried out by Dr. Rudolf Maibach.

Chapter 4: PCR samples for sequencing were prepared by the candidate and sequenced by Mr. Howard Potter (Dept. of Molecular Pathology). Statistical analysis was carried out by Dr. John Pearson.

Chapter 5: Dr. Mik Black carried out normalization and analysis of the Ambion miRNA data and Dr. John Pearson analysed the data from the Agilent miRNA arrays. Mr. Les McNoe helped with setting up the miRNA array protocols and with troubleshooting.

2.4 Tissue microarray construction

As the tumour samples all originated from outside sources and needed to be returned in a timely manner and in order to conserve valuable tissue, we decided to construct a tissue microarray (TMA). Tumour cases were selected for TMA construction and the original H+E slides of these cases reviewed by a pathologist who defined the morphologically representative areas and circled them. The matching FFPE donor blocks were then identified. A recipient block was prepared using low melting point wax and the array was designed using an Excel spreadsheet. To construct the tissue microarray, the Beecher Tissue Microarrayer MT-1 (Beecher Instruments, Sun Prairie, WI, USA) was used. A hollow needle was used to remove tissue cores of 1.0 mm in diameter from regions of interest marked on H+E slides of paraffin-embedded tissues. In total, there were 166 samples from 94 HB cases. These comprised diagnostic biopsies and/or surgical specimens and adjacent normal tissue in many cases. These tissue cores were then inserted into a recipient paraffin block in a precisely spaced, array pattern. Two cores of normal kidney and two cores of breast carcinoma were inserted in one corner as a means of orientation. Multiple 4µm sections from this block were cut using a microtome, mounted on microscope slides and then analyzed by standard methods of histological analysis; H+E staining and immunohistochemistry.
2.5 Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissue. Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumours. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. In this study the presence of antibody binding was indicated with brown staining (DAB) against blue haematoxylin counter staining to orientate protein expression around the anatomical features.

2.5.1 Section preparation for IHC

4 µm sections from the TMA block were cut on a Leica microtome (Leica, Wetzlar, Germany) and mounted onto poly-L-lysine coated slides (Menzel-Glaser, Braunschweig, Germany). Serial sections were cut in ribbons and placed on a cold water-bath where they were separated into single sections. These sections were mounted onto slides by floating them out on a water-bath at 50°C. Mounted sections were dried in an oven at 45°C overnight and then stored until required.

Prior to immunohistochemical analysis, the sections were baked overnight at 60°C then deparaffinised and re-hydrated by serial immersion as described below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Tap water</td>
<td>held until required</td>
</tr>
</tbody>
</table>
2.5.2 Envision™ method of antibody staining
Prepared sections were transferred to phosphate buffered saline (IHC-PBS, see appendix). Slides were not allowed to dry out during this procedure. All incubation steps were carried out in a humid Perspex chamber (Award Plastics, Christchurch).

2.5.3 Control Slides
For each antibody analyzed, two control slides were included. These controls included a positive tissue control slide, used to confirm positive antibody detection, and a negative control slide where the primary antibody was omitted to identify the presence of non-specific secondary antibody-chromogen binding. The positive control tissue for β-catenin, E-cadherin, Alpha-fetoprotein, Ki-67 and Cyclin D1 was breast carcinoma and the positive controls for phosphorylated β-catenin was colon carcinoma.

2.5.4 Endogenous Enzyme Block
Endogenous peroxidase activity in the tissue was blocked with a 3% hydrogen peroxide solution (BDH, Poole, England) in tap water for 15 minutes at room temperature. Slides were washed in tap water and then transferred to IHC-PBS (phosphate buffered saline pH 7.4).

2.5.5 Antigen Retrieval
Heat-induced epitope-retrieval (HIER) was used to unmask antigens on the surface of tissues. This was carried out by heating the slides to 121°C in 0.01M citrate buffer pH 6.0 in a Pressure Cooker for 2 minutes at full pressure. Slides were subsequently cooled to room temperature in running water and transferred to IHC-PBS.

2.5.6 Normal Serum Block
Non-specific binding of the secondary antibody-chromogen substrate was blocked by incubating each slide with 1% normal goat serum (Dako, Glostrup, Denmark) diluted in antibody diluent (Dako) with 1% Bovine serum albumin for 20 minutes at room temperature. Excess serum was tipped off the section but not washed prior to addition of the primary antibody.
2.5.7 Primary Antibody
The following commercial primary antibodies were purchased from Dako: β-catenin, E-cadherin, Alpha-fetoprotein, Ki-67 and Cyclin D1. Tyrosine 654-phosphorylated β-catenin antibody was sourced from Abcam, Cambridge, UK. Slides were incubated with the primary antibody diluted with Dako Antibody Diluent to working concentrations of 1:250 for β-catenin, 1:50 for E-cadherin, α-fetoprotein, Ki-67 and Cyclin D1 and 1:25 for phosphorylated β-catenin. Slides were incubated for 1 hour at room temperature for all slides except for tyrosine-phosphorylated β-catenin, which was incubated overnight at 4°C. Unbound primary antibody was removed by gentle washing in two changes of PBS/Tween and two washes with PBS. All antibodies were optimized for use in IHC using breast tumour and multi-tumour control slides kindly donated by the Department of Anatomical Pathology, CDHB, Christchurch and the appropriate positive and negative controls were used.

2.5.8 Labelled Polymer-Horse Radish Peroxidase (HRP)
Slides were incubated with Envision®+ Dual Link System HRP (Dako K1491) for 30 minutes at room temperature. This was followed by gentle washing through IHC-PBS/Tween as before.

2.5.9 Substrate-Chromogen Detection
Excess buffer was decanted from the slides, which were then treated with DAB+ working solution (Dako K3468). Chromogen binding to the antibody resulted in a brown stain, and optimization experiments determined that a four minute incubation was sufficient for all antibodies. The slides were then washed in running tap water.

2.5.10 Haematoxylin Counter Staining
Slides were counter stained with Haematoxylin using a program on the Leica Automated staining instrument in the Anatomical Pathology department. This program also dehydrates the slides through grades of Ethanol and clears the slides in Xylene. The slides were then mounted with DPX and coverslipped using the automated coverslipper also in the Anatomical Pathology department. Slides were allowed to dry fully before examination by light microscopy under bright field
illumination (Olympus BX50) and images were digitally acquired using a Leica digital camera (DFC420).

2.5.11 Analysis of Immunohistochemistry
The tissue array was made in duplicate and sections from the duplicate block were also stained with the antibodies in question and the results collated. The stained sections were separately evaluated by two pathologists (Dr Carina Miles and Dr Clinton Turner). The staining intensity was noted but not factored, as differing age of donor blocks and variation in fixation methods can impact on staining intensity. Subcellular distribution and relative expression levels of the proteins in question were evaluated in each HBL tumour or normal tissue core and given a score. For β-catenin, the tumour tissue was scored into negative, normal membranous, diffuse or focal cytoplasmic and diffuse or focal nuclear staining. E-Cadherin was reported as normal membranous, aberrant loss of staining or heterogenous staining. Cyclin D1 was reported as either positive (present) or negative (absent) in tumour cells. Ki67 nuclear positivity was counted as a percentage of total tumour cells and results were grouped into 0-10% or >10% positive. α-Fetoprotein was reported as either focal or diffuse staining of tumour cells. Staining for tyrosine 654-phosphorylated β-catenin (Y654-β-catenin) was scored as negative, cytoplasmic and/or nuclear staining.

2.6 RNA extraction from paraffin-embedded tumour tissue
Representative areas of tumour were identified on H+E slides by pathologists and a 1mm tissue core removed from corresponding areas on paraffin blocks. The RNA was extracted using RecoverALL™ Total Nucleic Acid Isolation kit (Ambion, Austin TX, USA). This method was also used to extract RNA from paraffin-embedded mouse liver control tissue.

2.6.1 Deparaffination
1ml of xylene was added to each tissue core sample in a 2ml eppendorf tube, vortexed and incubated in a heating block at 55°C for 10 minutes. The tubes were spun at 10,000 x g for 5 minutes to pellet the tissue. The xylene was removed and discarded into waste xylene container. The first step was repeated once more. 1ml of 100% ethanol was added to each sample to wash the tissue. The samples were mixed by
vortexing and spun for 2 minutes and the ethanol removed by aspiration. This step was repeated once more. The samples were centrifuged for a further 20 seconds and a fine-bore pipette tip used to remove any residual fluid. The tissue pellets were allowed to air-dry with the cap open.

2.6.2 RNA extraction

10µl of Proteinase K was mixed with 100µl of Proteinase K digestion buffer for each sample and this was added to the tissue sample. The samples were incubated for 3 hours at 37°C flicking the tubes periodically during the incubation. The samples were centrifuged for 1 minute to pellet the insoluble debris and the clear supernatant was transferred to a newly labelled tube. Approximately 60µl of elution solution was preheated per sample to 75°C on a heating block. A micro-filter cartridge and RNA elution tube was labelled for each sample. 200µl RNA extraction buffer was added to each sample and vortexed followed by 160µl ethanol to each sample. 235µl of each sample was transferred to a filter cartridge assembly and centrifuged for 1 minute. The remaining sample was transferred to the corresponding filter and spun for 1 minute. The filtrate was discarded and the cartridge replaced in the collection tube. 180µl of wash solution 1 was applied to the filter and centrifuged for 1 minute. A further 180µl of wash solution 2/3 was added to the filter cartridge and centrifuged for 1 minute. This step was repeated, the filtrate discarded and the filter cartridge replaced into the waste collection tube. The filter was dried by centrifugation for 2 minutes. The filter cartridge was transferred to a micro RNA elution tube. 20µl of preheated elution fluid was pipetted onto the centre of the filter, incubated at room temperature for 1 minute and centrifuged for 1 minute. A further 20µl of elution fluid was pipetted onto the filter and centrifuged for 1 minute giving an elution volume of approximately 40µl. The samples were stored at -80°C until further use.

2.6.3 DNase I treatment

2µl of 10X DNase buffer and 1µl DNase I was added to each sample and mixed. The samples were incubated at 37°C for 30 minutes. 3µl of DNase inactivation reagent was added to each tube and mixed well. The samples were incubated at room temperature for 2 minutes flicking the tube frequently. The tubes were centrifuged for
2 minutes to pellet the DNase inactivation reagent. The RNA-containing supernatant was transferred to a new RNAse-free tube and stored at -80°C.

2.7 RNA extraction from cell lines and fresh tissue

2.7.1 Organic extraction from Cell Lines
Total RNA was extracted from the HuH-6 and Huh-7 cell lines using the MirVana™ PARIS™ Protein and RNA Isolation kit (Ambion). Firstly ~10^6 cells were harvested using the method described in 2.6.4. 300µl of ice-cold Cell Disruption buffer was added to each sample and vortexed vigorously to lyse the cells. An equal volume of 2x Denaturing Solution was added and the samples mixed and incubated on ice for 5 minutes. 600µl of acid-phenol:chloroform was added to each sample and vortexed for 1 minute to mix. The samples were then centrifuged at 10,000g for 5 minutes to separate aqueous and organic phases. The aqueous phase was carefully removed to a clean tube and the volume noted.

2.7.2 Organic extraction from fresh tissue stored in RNAlater™
Using the MirVana™ PARIS™ Protein and RNA Isolation kit (Ambion) 0.01g of tissue was taken from each mouse liver sample stored in RNAlater™ (Ambion). This was added to 100µl of lysis buffer and homogenized using a micropestle before adding 10µl miRNA homogenization additive and mixing vigorously. The tubes were incubated on ice for 10 minutes. One volume (110µl) of acid-phenol:chloroform was added from the bottom phase and vortexed. The tubes were then centrifuged at 10,000 x g for 5 minutes to separate the aqueous and organic phases. The aqueous upper phase was carefully removed to a clean tube and the volume noted.

2.7.3 Final RNA extraction for cell lines and fresh tissue
RNAse-free dH₂O was preheated to 95°C. 1.25 times the aqueous phase volume of 100% ethanol was added and mixed and the mixture pipetted onto a filter cartridge placed in a collection tube. This was centrifuged to pass the mixture through the filter and the flow-through was discarded. The filter was washed with 700µl of miRNA Wash Solution 1 and twice with 500µl miRNA Wash Solution 2/3, discarding the flow-through each time. The filter cartridge was placed in a fresh tube and 100µl of
preheated dH₂O applied to the center of the filter and centrifuged to collect purified RNA. The samples were stored at -80°C.

2.8 RNA measurement for purity and concentration
Ideally a platform such as the Bioanalyser2100 from Agilent would be used to quantify and measure RNA quality, but such a system was not available at our location. The Nanodrop 2000 (Thermo Scientific, Wilmington, MA, USA) was therefore used to measure quality and quantity of RNA extracted. The nucleic acid measurement option was selected on Nanodrop2000 spectrophotometer. RNA measurement was selected. A blank measurement was made with 1µl elution fluid. 1µl of RNA sample was loaded onto the Nanodrop platform and the optical density measured at 260 and 280nm. The ratio of the two is calculated and should be between 1.9 and 2.1 for pure RNA. The concentration of RNA is given in ng/µl.

2.9 RT-PCR to amplify a housekeeping gene from extracted RNA
We used reverse-transcription polymerase chain reaction (RT-PCR) to amplify a 206bp region of the GAPDH housekeeping gene from each of our HB samples. This is to ensure that the RNA is sufficiently intact to amplify a similar size or smaller product from our gene of interest. The QIAGEN one-step kit was used to perform RT-PCR (QIAGEN, Dusseldorf, Germany). All reagents, RNA samples and primers were thawed on ice and tubes were vortexed and centrifuged briefly before use. A 6µM stock solution of the gene specific primers for GAPDH was made and aliquoted (primers are used at 0.6µM). A master mix was prepared by adding the following volume of each reagent for each sample multiplied by the number of samples plus one (e.g. for 10 samples multiply the volume of each reagent by 11).

<table>
<thead>
<tr>
<th></th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>25µl</td>
</tr>
<tr>
<td>5x buffer</td>
<td>10µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2µl</td>
</tr>
<tr>
<td>primer 1</td>
<td>5µl</td>
</tr>
<tr>
<td>primer 2</td>
<td>5µl</td>
</tr>
<tr>
<td>RT-PCR enzyme</td>
<td>2µl</td>
</tr>
</tbody>
</table>
The master mix was vortexed, centrifuged briefly and 49µl of the mix pipetted into each 200µl PCR tube. Keeping the tubes on ice, 1µl of sample RNA, H₂O (negative control), or commercial fetal liver RNA (positive control) was added to the mix. Samples were reverse transcribed and amplified on a DNA Engine Thermal Cycler (BioRad, Hercules, CA, USA). The annealing temperature was 54°C.

The primers used for reverse transcription of GAPDH are as follows:

**Forward:** 5' GGAGTCAACGGATTTGGT 3'  
**Reverse:** 5' GTGATGGGATTTCCATTGAT 3'

The program for RT-PCR of GAPDH was as follows:

<table>
<thead>
<tr>
<th>Reverse transcription</th>
<th>30mins</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st strand synthesis</td>
<td>15mins</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>1min</td>
<td>94°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>30sec</td>
<td>54°C</td>
</tr>
<tr>
<td>Extension</td>
<td>30sec</td>
<td>72°C</td>
</tr>
<tr>
<td>Final extension</td>
<td>10mins</td>
<td>72°C</td>
</tr>
<tr>
<td>Storage</td>
<td></td>
<td>4°C</td>
</tr>
</tbody>
</table>

### 2.10 Agarose gel electrophoresis of RT-PCR products

A 1.5% agarose gel was used to separate amplified fragments. 0.8g agarose LE (Roche Diagnostics) was added to 50ml of 1x Tris-borate EDTA buffer (TBE 90mM Tris-borate, 2mM EDTA) and dissolved in a microwave. Allowing the gel to cool slightly, 10µl of ethidium bromide was added and the gel poured into a gel-casting tray. A gel comb was inserted at the anode and the gel allowed set for 30 minutes before removing the comb and placing the gel plate into the electrophoresis chamber and immersing in 0.5x TBE running buffer.

10µl of sample was mixed with 2µl of 6x loading buffer (Fermentas, Burlington, Ontario, Canada) containing bromophenol blue for each sample. 10µl of Trackit™ 25bp DNA ladder (Invitrogen) was loaded into the first well followed by RT-PCR samples. The power pack was connected so that the DNA migrated towards the
positive electrode and run at 60-80V until the dye had migrated 2/3 of the gel towards the anode. PCR products were visualized under UV light and photographed on the GelDoc 2000 molecular imaging system using the QuantityOne v4.6.5 software (BioRad Laboratories).

2.11 CTNNB1 mutation detection

Samples with the following quality parameters were analysed for CTNNB1 gene mutations: Optical density ratio 260/280 of 1.8 – 2.2 and RNA concentration of >20ng/ul using a nanodrop spectrometer and amplification of GAPDH. A 150bp region of the CTNNB1 gene was amplified that includes the β-catenin regulatory region of exon 3 (codons 32-45) using the following primer pair P1 and P2:

P1: 5’ GATTTGATGGAGTTGGACATGG 3’
P2: 5’ TCTTCCTCAGGAGTTGGACATGG 3’

To detect deletions in exon 3 of CNTNB1, RT-PCR was carried out using the primers P3 and P4 that flank the entire exon:

P3: 5’ TACAACCTGTTTTGAAAATCCAGCGTGGAC 3’
P4: 5’ TCGAGTCATTGCATACTGTCC 3’

Samples were reverse transcribed and amplified using One-Step RT-PCR kit (QIAGEN) on a DNA Engine Thermal Cycler (BioRad) as outlined above. The annealing temperature was 52°C. RT-PCR products were visualised on a 1.5% agarose gel with ethidium bromide as outlined previously.

2.11.1 CTNNB1 mutation analysis of cell lines

CTNNB1 mutation detection was carried out as outlined above in 2.9 for the two cell lines.

2.11.2 Purification of RT-PCR products

Amplified RT-PCR products were purified using QIAquick PCR purification kit (QIAGEN). Five volumes of PB buffer was added to each sample i.e. 200µl to 40µl sample, mixed and applied to a QIAquick column. After centrifugation for 1 minute the flow-through was discarded. 750µl of PE buffer was then added to the column,
centrifuged, flow-through discarded and centrifuged again to dry the filter. The column was placed in a new 1.5ml collection tube and 50µl EB buffer was added to the filter. After incubation for 1 minute at room temperature, the tubes were centrifuged for 1 minute to elute the purified DNA. The samples were stored at -80°C until further use.

2.11.3 DNA sequencing of amplified CTNNB1 fragment

Cycle sequencing was carried out using ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). 20ng in 1µl of RT-PCR product was used with 1 x sequencing buffer (Applied Biosystems), 5pmol primer, 0.5µl BigDye Terminator v3.1 (Applied Biosystems) in a total volume of 10µl. Thermal cycling conditions were as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time/Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st strand synthesis</td>
<td>1 min 96°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10 sec 96°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>10 sec 50°C 25 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>2 min 60°C</td>
</tr>
</tbody>
</table>

The sequencing reaction products were purified with Sephadex G50 DNA grade resin (GE Healthcare, Chalfont, St. Giles, UK). The Sephadex was hydrated in excess ddH₂O then 500µl pipetted into each well of a Unifilter 800 96-well microtitre plate (Whatman Inc., Clifton, NJ, USA). The resin was dehydrated by centrifugation at 750 x g for 5 minutes on top of a 96-well collection plate. The resin plate was then placed on top of a 96-well PCR microplate (Axygen Scientific Inc., Union City, CA, USA). Each sequencing reaction was then mixed with 10µl of ddH₂O and pipetted onto the Sephadex resin plate. This was then centrifuged at 750 x g for 5 minutes to collect the purified PCR products in the lower plate.

Sequencing products were resolved by capillary electrophoresis on an ABI 3130 XL-Avant genetic analyser (Applied Biosystems) and all mutation were verified by sequencing the sense and anti-sense strands. Mutation analysis was carried out using Variant™ Reporter Software (Applied Biosystems) by comparing sequence traces to the wild type CTNNB1 sequence. The software also carries out quality analysis on the sequence traces.
2.12 Tissue Culture

2.12.1 Routine culture of HuH-6 and HuH-7 cells
All cell culture reagents were sourced from Invitrogen. Human hepatoblastoma cells, HuH-6 (Japanese Collection of Research Bioresources (JCRB), Osaka, Japan) were routinely maintained in minimum essential media (MEM) containing 10% Fetal Calf Serum (FCS) and penicillin/streptomycin. The human hepatoma cell line HuH-7 (JCRB) was cultured in Dulbecco’s Minumum Essential Media (D-MEM) with 10% FCS and penicillin/streptomycin (pen/strep). All tissue culture work was carried out in a tissue culture hood with especial consideration given to aseptic technique. Culture media, FCS and penicillin-streptomycin were warmed to 37°C in a water bath. The FCS was mixed by pipetting and 50mls were added to the media. 5.5mls of pen/strep were also added. 20ml of media was added to each culture flask. Vials of cells were removed from liquid nitrogen and thawed to 37°C in a water bath and the contents of each added to a flask containing media. The cell cultures were then placed in an incubator with 5% CO$_2$ at 37°C.

2.12.2 Passaging of cells
Cells were passaged when approximately 80% confluent as determined by microscopic examination. Culture media, trypsin (Invitrogen) and PBS (Invitrogen) were prewarmed to 37°C. Media was removed carefully from culture flasks using a vacuum pipette and washed with two 10ml changes of PBS. To lift the adhesive cells from the culture flask, 1.5ml of trypsin was added and swirled gently over the cells. The trypsin was left on the cells for 5 minutes and then 8.5ml of media was added to inhibit the trypsin giving a total volume of 10ml. 15ml of fresh medium was added to two new flasks and labelled P2 (passage 2). The cell suspension was triturated to remove clumps and 5ml of this added to the two new flasks to give a 1:2 split.

2.12.3 Cryogenic storage of cell lines
After two passages of cell lines three flasks of cells from each cell line were frozen to ensure ongoing stocks of cells for future use as follows. The cells were washed and trypsinized and media added to 10ml volume as above ensuring a single cell solution.
The solution was transferred to a 15ml Falcon tube and centrifuged for 5 minutes at 100rpm. Sufficient media containing 20% FCS and dimethyl sulfoxide (DMSO) (Thermofisher Scientific) to a final concentration of 10% was made up to freeze each aliquot in 1ml media. The supernatant was removed from the cells and they were resuspended in 1ml of the prepared media and transferred to a cryotube. The cryotubes were placed in a NALGENE® Labware “Mr. Frosty” (Thermo Fisher Scientific, Waltham, MA, USA) with isopropyl alcohol overnight at -80°C to gradually freeze the cells and placed in liquid nitrogen the following day.

2.13 Timecourse experiment

2.13.1 Seeding of cells for timecourse experiment
Cells were grown to ~80% confluency and then trypsinized (1.5ml) for 5 minutes and 8.5ml media added as described previously. A drop of the single cell solution was placed in a haemocytometer and the cells counted. For the timecourse experiments 1 million cells were needed in each of five plates for each cell line in a 10ml volume. The cells were allowed to equilibrate for 24 hours in 5% CO₂ at 37°C.

2.13.2 Serum starvation of cell lines
Cells were washed twice with prewarmed PBS. The media was replaced with 10ml of serum-free media containing pen/strep. This is to ensure that growth factors present in the serum do not interfere with the experiment. The cells were incubated for 24 hours.

2.13.3 Timecourse experiment with HGF
100µl dH₂O was added to lyophilized human recombinant HGF (Gibco®), mixed and centrifuged briefly. This gives a 0.1mg/ml solution. 5µl of HGF was added to each plate at each timecourse point (giving a concentration of 50ng/ml) except to T0, the negative control.

2.13.4 Harvesting cells
Keeping plates on ice, the media was removed from cells with a vacuum pipette and rinsed twice with cold PBS. The PBS was removed. The cells were scraped from the plate surface using 1ml fresh PBS into 1.5 ml tubes. These were centrifuged for 5
mins at 450g and the supernatant was decanted and discarded. The packed-cell volume (PCV) was noted.

2.14 Extraction of Nuclear and Cytoplasmic proteins following HGF treatment

The CelLytic™ NuCLEAR™ extraction kit (Sigma®, Missouri, USA) was used to extract cytoplasmic and nuclear proteins. All buffers were prepared prior to harvesting cells. All reagents and buffers were cooled prior to use and work was carried out on ice.

2.14.1 Extraction buffers

- To make up 50µl of 0.1M DTT
  - 5µl of 1M DTT
  - + 45µl dH₂O

- To make up 3ml of 1x lysis buffer
  - 300µl 10x buffer + 2.7ml H₂O
  - + 30µl 0.1M DTT
  - + 30µl Protease inhibitor cocktail

2.14.2 Cytoplasmic protein extraction

500µl of 1x lysis Buffer (with DTT and protease inhibitors) was added to 100µl of PCV and resuspended gently. This was incubated on ice for 15 minutes allowing the cells to swell (checking under a microscope that they are not lysed). 10% IGEPAL solution was added to a final concentration of 0.6% (6µl/100µl mix) and vortexed vigorously 10 seconds. The tubes were centrifuged for 30 seconds at 11,000 x g and the supernatant transferred to a fresh cooled tube. 20µl aliquots were removed at this stage for BCA assay and the samples stored at -80°C.

2.14.3 Nuclear protein extraction

5µl of 0.1M DTT and 5µl protease inhibitor was added to 490µl of the extraction buffer. Each nuclear pellet was resuspended in ~ 70µl of this extraction buffer. The
tubes were mounted on a vortex mixer and agitated at a medium–high setting for 15–30 minutes. The tubes were then centrifuged for 5 minutes at 20,000g. The supernatant was transferred to a clean chilled tube removing 20µl aliquots for BCA assay and freezing at -80°C.

2.15 BCA Protein assay

The lysate protein concentrations were determined by bicinchoninic acid (BCA) protein assay using bovine serum albumin (BSA) as a standard (Pierce, Rockford, IL, USA) using the microplate protocol. The albumin standards were prepared by using the same diluent as used for samples i.e. extraction buffers. The working range is 20-2000µg/ml

A  2000µg/ml
B  1500µg/ml
C  1000µg/ml
D  750µg/ml
E  500µg/ml
F  250µg/ml
G  125µg/ml
H  25µg/ml
I  0µg/ml (blank)

The BCA working reagent was prepared as follows:

\[
\text{(stds + unknowns) x (replicates (2)) x (200µl)} = \text{volume of working reagent required}
\]

\[
e.g. \ 23 \times 200µl = 4600µl = 4.6ml
\]

make up 5ml solution A + 100µl solution B (50:1 A:B)

25µl of each standard or unknown was pipetted into a microplate well (or 10µl x 2 replicates) and 200µl of working reagent was added. The microplate was mixed on a plate shaker for 30 seconds, covered and incubated for 30 minutes at 37°C. The plate
was cooled to room temperature and the absorbances read at 562 nm on a SpectraMax 190 spectrophotometer using SoftMax version 5.9 software.

2.16 Protein Methods

2.16.1 Gel separation of extracted proteins
Approximately 10 µg of protein sample were run on NuPAGE 4-12% BisTris gels (Invitrogen™ Corporation, CA, USA) with MES-SDS buffer (Invitrogen™) using the Xcell SureLock™ Mini-Cell (Invitrogen™). 10 µg protein samples, as determined by BCA assay, were made up to 10 µl with dH2O and 2 µl of 6x loading dye (containing bromophenol blue) was added. The samples were mixed, spun briefly and heated at 99°C for 5 minutes to denature the proteins. The samples were then transferred to ice and immediately loaded on the gel. The protein marker used was Precision Plus Protein™ Standards (BioRad) and 12 µl aliquots of this were pipetted into the first, last and centre wells of the gel. The gel was electrophoresed at 200 V for 35 minutes until the bromophenol blue reached the bottom of the gel. The gel was then removed carefully from the electrophoresis plates and trimmed with a scalpel to the size of the membrane.

2.16.2 Western Blotting of separated proteins
The iBlot® Gel Transfer Device (Invitrogen™) was used for western blotting of proteins. The gel was placed in contact with the nitrocellulose membrane and cathode and anode stacks as illustrated below. The blotting system was run for 7 minutes to transfer the proteins from the gel to the membrane. The membrane was then removed and trimmed to size and one corner marked with a scalpel for orientation.
Figure 2.1: Schematic diagram of iBlot® Dry Blotting system showing the flow of current.

2.16.3 Antibody detection of specific proteins

Blocking buffer and antibody diluent were made up as follows: 5% BSA (Gibco® Invitrogen) in 1x TBS with 0.1% Tween 20. This can be stored for 24 hours at 4°C. The membrane was placed into 50ml blocking buffer for one hour at room temperature with gentle rocking. The membrane was then transferred into antibody diluted in blocking buffer to the correct dilution (Table 2.3.). All primary antibodies were sourced from Abcam and incubated overnight at 4°C with gentle rocking. The filters were then washed twice with TBS-T for 10 minutes and twice with TBS for 10 minutes. The immunoblots were then incubated for 1 hour with the appropriate secondary antibodies (mouse/rabbit) at 1:10,000 coupled to horseradish peroxidase with gentle rocking and washed as described above. This was followed by exposure to ECL plus chemiluminescence reagents (GE Healthcare) for 5 minutes. The proteins were visualized by exposing Amersham Hyperfilm ECL (GE Healthcare) to the immunoblot for varying times before development.
Table 2.3. Antibodies used in Western blot analysis showing working dilutions and antibody species.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y654 β-catenin</td>
<td>1:150</td>
<td>Rabbit</td>
</tr>
<tr>
<td>β-catenin</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>TBP</td>
<td>1:3000</td>
<td>Mouse</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:2000</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>

2.16.4 Stripping filters for reprobing
The filters were stripped with a mild stripping buffer containing 1.5% glycine, 0.1% SDS and 1% Tween-20 twice for 10 minutes followed by two 10 minute-washes in TBS and two washes in TBS-Tween for 5 minutes each. This sufficiently removed antibody and label and allowed for reprobing with different antibodies.

2.17 Statistical Methods

2.17.1 Statistical analyses used in Chapter 3
Dr Rudolf Maibach, Consultant Biostatistician for SIOPEL, carried out the statistical analysis for Chapter 3. He first constructed frequency tables and then made a comparison of assayed versus non-assayed SIOPEL-3 patients in order to check if the assayed population is similar to the non-assayed in terms of presentation at diagnosis and risk factors. This ensured that there was no bias among the assayed cohort and therefore any correlation with event-free survival (EFS) seem genuine. For analysis of histologic type and subtype, it was necessary to simplify the categorization for correlation with biomarkers to create less sparse tables. This was achieved by collapsing some categories. Results were analysed with SAS® software v9.2 (SAS Institute Inc., Cary, NC, USA). For correlation of histology type and biomarkers, small categories were omitted and Fisher’s Exact test was used. For survival analysis, the LIFETEST procedure was used and the Log-Rank test was used to test the homogeneity of survival curves over strata. A P value of <0.05 was considered statistically significant.
2.17.2 Statistical analysis in Chapter 4 + 5
The “R” statistical package was used for analyses carried out in Chapter 4 and 5. R is a language and environment for statistical computing and graphics that was developed at Bell Laboratories (now Lucent Technologies) by John Chambers and colleagues and is available as a free internet download. R provides a wide variety of statistical (linear and nonlinear modelling, classical statistical tests, time-series analysis, classification, clustering) and graphical techniques. The Fisher Exact test was used for to analyse the statistical significance of correlations and a \( P \)-value of <0.05 was considered significant. Dr John Pearson, Consultant Biostatistician with the University of Otago, Christchurch, carried out statistical analysis for Chapters 4 and 5.

2.18 MicroRNA methods

2.18.1.1 miRNA isolation from total RNA using FlashPAGE™ Fractionator
30\( \mu \)l of total RNA sample extracted from either FFPE or fresh liver tissue (Section 2.5) was mixed with 30\( \mu \)l of loading buffer and incubated at 95°C for 2 minutes and then placed on ice. Meanwhile the FlashPAGE™ Fractionator (Ambion) instrument was set up by pipetting 250\( \mu \)l of lower running buffer into the chamber. The FlashPAGE™ gel was removed from the fridge, blotted dry and carefully placed into the lower chamber of the fractionator, avoiding bubbles at the interface. 250\( \mu \)l of upper running buffer was pipetted into the gel cartridge. A sample was loaded onto the gel surface and the lid closed making sure the upper electrode is immersed. The gel was electrophoresed at 75V until the blue dye just exits the gel (~12 minutes). The gel cartridge was removed and discarded. The lower running buffer that contains the microRNA was carefully pipetted into a new tube.

2.18.1.2 FlashPAGE™ Reaction Cleanup
A filter cartridge was prewetted for each samples by adding 500\( \mu \)l of wetting solution and centrifuging at 2500 x g for 1 minute and discarding the flow-through. 135\( \mu \)l of filter binding additive and 1.1ml ethanol was added to each sample and mixed. The solution was drawn through the filter by centrifugation. The filter was then washed twice with 500\( \mu \)l wash solution and dried by centrifugation at 10000 x g for 1 minute.
The filter was then placed in a fresh collection tube and 20µl of dH2O, preheated to 95°C, was pipetted onto the filter and incubated at 65°C for 10 minutes. The filter cartridge was then centrifuged at 10,000 x g for 1 minute and the final step repeated with a further 20µl aliquot of heated dH2O. The RNA concentration was measured and the samples stored at -80°C.

2.18.2 mirVana™ miRNA Probe Set

The mirVana™ miRNA Probe Set (Ambion) was used to generate spotted oligonucleotide arrays on glass slides. These were prepared by Les McNoe at the Otago Genomics Facility. The probe set contains human, mouse and rat miRNA probes from the miRNA Registry: microrna.sanger.ac.uk/sequences/index.shtml. Additionally, the mirVana™ miRNA probe set contains probes to a set of human miRNA that were novel at the time of printing.

2.18.3 mirVana™ miRNA labeling

2.18.3.1 PolyA tailing of miRNA

The mirVana™ miRNA labeling kit (Ambion) was used to prepare miRNA samples for microarray analysis. Samples prepared using the flashPAGE™ protocol were dried in a Speedivac for 1 hour and resuspended in 3µl dH2O. The mirVana™ positive control was prepared by diluting 1µl in 499µl dH2O. The tailing mixture was prepared keeping all reagents on ice as follows:

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>miRNA sample</td>
</tr>
<tr>
<td>1</td>
<td>diluted positive miRNA control</td>
</tr>
<tr>
<td>10</td>
<td>2x Poly (A) polymerase reaction buffer</td>
</tr>
<tr>
<td>2</td>
<td>25mM MnCl₂</td>
</tr>
<tr>
<td>2</td>
<td>10x Amine-NTP mix</td>
</tr>
<tr>
<td>2</td>
<td>Poly (A) polymerase</td>
</tr>
<tr>
<td>20</td>
<td>Total Volume</td>
</tr>
</tbody>
</table>

The tailing mixture was vortexed, spun briefly and incubated at 37°C for 2 hours.
2.18.3.2 Post-tailing cleanup
10µl of miRNA carrier and 350µl of binding/wash buffer was added to each sample and mixed. The samples were incubated at room temperature for 5 minutes before pipetting sample onto the miRNA cartridge in a collection tube. The tubes were centrifuged for 15 seconds at 10,000 x g and washed twice with 300µl of binding/wash buffer. The filter was dried by centrifugation for 1 minute at 10,000 x g. The filter was then placed in a fresh collection tube and the tailed miRNA was eluted with 15µl of elution solution, preheated to 95°C. This was incubated for 10 minutes at 65°C and centrifuged for 15 seconds. The elution step was repeated with a further 15µl aliquot of elution solution. Samples were stored overnight at -20°C and the following day, dried down in a speedivac for 30 minutes.

2.18.3.3 Labeling tailed miRNA with amine-reactive dye
4µl of DMSO was added to either Cy™3 or Cy™5 AlexaFluor dyes (Amersham, GE Healthcare). In our array study the reference sample was always labeled with Cy™3 and the test labeled with Cy™5 so that any probe-specific dye bias not removed by normalization was consistent across all arrays. 7µl dH₂O was added to each miRNA sample and mixed to resuspend. Dye, miRNA sample and 9µl of coupling buffer were mixed together and spun briefly, taking care to keep the tube in the dark to avoid photobleaching. The sample was incubated for 1 hour at room temperature before adding 4.5µl 4M hydroxylamine and incubating for a further 15 minutes at room temperature to quench the fluorescence.

2.18.3.4 Post-labeling miRNA cleanup
350µl of binding/wash buffer was added to each sample, mixed and incubated for 5 minutes at room temperature. Both reference and test samples were pipetted onto a single filter cartridge in a collection tube and centrifuged for 15 seconds at 10,000 x g. The filter was washed twice with 300µl wash buffer, dried by centrifugation for 1 minute and transferred to a new collection tube. 22µl of dH₂O, preheated to 95°C, was pipetted onto the filter and incubated at 65°C for 10 minutes before centrifugation for 1 minute at 10,000 x g to elute the miRNA.
2.18.3.5 Prehybridization of array slides
The prehybridization buffer was made up by adding 20ml of 20x SSC, 5ml of 10% SDS and 1g of BSA and making up to 100ml with dH₂O. It was filtered through a 0.2µm filter and preheated to 42°C in a 50ml falcon tube. The back of the array slide was marked with a diamond pen for orientation prior to prehybridization at 42°C for 45 minutes. The slide was then rinsed in four changes of dH₂O before centrifugation for 10 minutes at 800 x g to dry the slide.

2.18.3.6 miRNA array hybridization
3x miRNA hybridization byffer was heated to 65°C for 5 minutes and mixed well. The microarray coverslip was cleaned with 70% ethanol using a lint-free wipe to remove all dust particles and placed on the array slide with the raised edge facing downwards. The miRNA sample was prepared by bringing the volume to 20µl with dH₂O and adding 10µl of 3x hybridization buffer. The sample was heated to 95°C for 3 minutes to denature the RNA, centrifuged briefly and incubated in the dark at room temperature for 1 minute to cool. The array slide was placed in the hybridization chamber and the sample carefully pipetted under the coverslip. To prevent dehydration, dH₂O was pipetted into the chamber pools before sealing chamber securely. The hybridization chamber was placed in the bottom of a water bath at 42°C overnight in the dark.

2.18.3.7 Washing of miRNA arrays

<table>
<thead>
<tr>
<th></th>
<th>20x SSC</th>
<th>10% SDS</th>
<th>dH₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1</td>
<td>5ml</td>
<td>0.5ml</td>
<td>44.5ml</td>
</tr>
<tr>
<td>Solution 2</td>
<td>2.5ml</td>
<td>0.5ml</td>
<td>47ml</td>
</tr>
<tr>
<td>Solution 3</td>
<td>1.25ml</td>
<td>-</td>
<td>48.75ml</td>
</tr>
<tr>
<td>Solution 4</td>
<td>0.25ml</td>
<td>-</td>
<td>49.75ml</td>
</tr>
</tbody>
</table>

The four post-hybridization wash solutions were made up as outlined above and filtered through a 0.2µm filter. Slides were washed for 1 minute in each solution, allowing the coverslip to lift off in the first wash solution and ensuring the slide does
not dry out between washes. The slide was then dried by centrifugation for 15 minutes at 800 x g.

2.18.3.8 Scanning Ambion miRNA array slides
Slides were scanned at 647nm (Cy™5 Dye) and 555nm (Cy™3 Dye) in a GenePix 4000B Scanner (Axon Instruments, Foster City, CA, USA). Median fluorescent data was collected for each wavelength for each miRNA probe. The fluorescent data was then filtered, normalised and analysed using BRB Array Tools (Biometric Research Branch, National Cancer Institute, MD, USA).

2.18.4 NCode™ miRNA amplification
Due to the low yield of miRNA from our HB samples and the need to conserve remaining RNA, it was decided to employ a means of miRNA amplification. The NCode™ miRNA Amplification System from Invitrogen™ was used as this allowed us to use as little as 5ng of starting RNA for array analysis while still preserving the relative abundance of individual miRNAs in the original sample.

2.18.4.1 Isolation of small RNA using PureLink™ miRNA isolation kit
Prior to miRNA amplification, the fraction of RNA containing small RNAs was isolated from the total RNA sample using the PureLink™ miRNA isolation kit (Ambion). Aliquots containing ~500ng of total RNA were pipetted into 1.5ml tubes and dried in a vacuum centrifuge for 60 minutes before resuspending in 300µl of Binding buffer. A further 300µl of ethanol was added and mixed and the entire sample was pipetted onto a spin cartridge in a collection tube and centrifuged for 1 minute at 10,000 x g. The cartridge was discarded and 700µl of ethanol added to the flow-through and mixed. The mixture was added to a new cartridge and centrifuged for 1 minute at 10,000 x g and the flow-through discarded. The filter was washed twice with 500µl Wash Buffer before placing the filter in a wash tube and dried by centrifugation for 3 minute at 10,000 x g. The filter was then placed in a 1.7ml recovery tube, 50µl of dH2O added and incubated at room temperature for 1 minute before centrifugation for 1 minute at 10,000 x g to recover the enriched small RNA fraction. The RNA concentration in the samples was measured using the Nanodrop spectrophotometer as outlined above.
2.18.4.2 PolyA tailing of miRNA

30ng of miRNA from section 2.5 was pipetted into a 1.5ml tube and the volume made up to 18µl with dH₂O. Dilute samples were concentrated in a vacuum centrifuge prior to use. The following master mix was prepared for the appropriate number of samples and 7µl of the master mix added to each sample. The tubes were mixed, spun briefly and incubated for 15 minutes at 37°C.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10x miRNA reaction buffer</strong></td>
<td>2.5µl</td>
<td></td>
</tr>
<tr>
<td><strong>25mM MnCl₂</strong></td>
<td>2.5µl</td>
<td></td>
</tr>
<tr>
<td><strong>Diluted ATP</strong></td>
<td>1µl</td>
<td></td>
</tr>
<tr>
<td><strong>PolyA polymerase</strong></td>
<td>1µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>7µl</td>
<td></td>
</tr>
</tbody>
</table>

2.18.4.3 Reverse transcription of tailed miRNA

Aliquots of Oligo(dT) primer were centrifuged briefly and thawed on ice and 2µl added to each sample and mixed. The samples were incubated at 65°C for 10 minutes and placed on ice for 2 minutes. A master mix was prepared containing the following components for the appropriate number of samples.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5x 1st strand buffer</strong></td>
<td>10µl</td>
<td></td>
</tr>
<tr>
<td><strong>0.1M DTT</strong></td>
<td>5µl</td>
<td></td>
</tr>
<tr>
<td><strong>10mM dNTP mix</strong></td>
<td>2.5µl</td>
<td></td>
</tr>
<tr>
<td><strong>RNase Out</strong></td>
<td>1µl</td>
<td></td>
</tr>
<tr>
<td><strong>Superscript III reverse transcriptase</strong></td>
<td>2µl</td>
<td></td>
</tr>
<tr>
<td><strong>DEPC-H₂O</strong></td>
<td>2.5µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20µl</td>
<td></td>
</tr>
</tbody>
</table>

25µl of master mix was added to each sample and mixed gently and incubated for 1 hour at 46°C. The reaction was stopped by adding 8.75µl 50mM EDTA to each tube and mixing. This was followed by an incubation for 30 minutes at 65°C. 12.5µl of 1M
Tris pH8.0 was added and mixed followed by an addition of 28.75µl of 1x TE buffer, giving a final volume of 100µl per sample.

2.18.4.4 Purification of tailed miRNA
A cDNA ultracentrifugation column was inserted into a collection tube for each sample and 100µl of sample pipetted into the column without touching the membrane. The column was centrifuged for 6 minutes at 13,000 x g and the flow-through discarded and the column replaced in the tube. 200µl of 1x TE buffer was added to the column, pipetted up and down and centrifuged for 6 minutes at 13,000 x g and the flow-through discarded. The previous step was repeated once, discarding the flow-through and tube. 5µl of 10mM Tris pH 8.0 was pipetted onto the filter and tapped to mix. The filter was then placed upside down in a new collection tube and centrifuged for 3 minutes to elute the concentrated DNA. The volume was brought up to 10µl with DEPC-H₂O.

2.18.4.5 Tailing of first-strand cDNA
Samples from the previous step were incubated at 80°C for 10 minutes before placing on ice. The following master mix was prepared for the appropriate number of samples.

<table>
<thead>
<tr>
<th>10x miRNA reaction buffer</th>
<th>2µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM dTTP</td>
<td>4µl</td>
</tr>
<tr>
<td>Terminal DT</td>
<td>2µl</td>
</tr>
<tr>
<td>DEPC-H₂O</td>
<td>2µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10µl</strong></td>
</tr>
</tbody>
</table>

After mixing and brief centrifugation, 10µl of master mix was added to each sample and incubated for 3 minutes at 37°C. The reaction was stopped by placing the samples at 80°C for 10 minutes. The samples were centrifuged briefly and allowed to cool to room temperature.
2.18.4.6 T-7 promoter synthesis

2µl of T7 template oligo was added to each sample and incubated at 37°C for 10 minutes. A master mix was prepared as outlined below and 3µl of the master mix added to each sample.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x miRNA reaction buffer</td>
<td>1µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1µl</td>
</tr>
<tr>
<td>Klenow enzyme</td>
<td>1µl</td>
</tr>
</tbody>
</table>

Total 3µl

The samples were mixed, briefly centrifuged and incubated at room temperature for 30 minutes. The reaction was stopped by placing the samples in a heating block at 65°C for 10 minutes before placing them on ice.

2.18.4.7 In Vitro transcription

The T7 RNA polymerase, 10x T7 reaction buffer and the four dNTPs were thawed to room temperature. The samples were incubated at 37°C for 10 minutes to re-anneal the DNA strands and the following master mix was prepared.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM ATP</td>
<td>1.5µl</td>
</tr>
<tr>
<td>100 mM CTP</td>
<td>1.5µl</td>
</tr>
<tr>
<td>100 mM GTP</td>
<td>1.5µl</td>
</tr>
<tr>
<td>100 mM UTP</td>
<td>1.5µl</td>
</tr>
<tr>
<td>10x T7 buffer</td>
<td>4µl</td>
</tr>
<tr>
<td>T7 RNA polymerase</td>
<td>7µl</td>
</tr>
</tbody>
</table>

Total 17µl

17µl of the master mix was added to each sample, mixed and centrifuged briefly before incubating at 37°C for 4 - 16 hours. The samples were then stored at -80°C.
2.18.4.8 Purification of amplified miRNA

Prior to array analysis of the miRNA samples, they were purified using the PureLink™ Micro-to-Midi™ Total RNA purification system (Invitrogen). The lysis buffer was prepared by adding 20µl of 2-mercaptoethanol to 2ml of lysis buffer. 42µl of the lysis buffer was added to 42µl of senseRNA from the previous step, followed by 42µl of ethanol. The sample was mixed well and pipetted onto an RNA spin cartridge and centrifuged for 15 seconds at 10,000 x g. The flow-through was discarded and the filter was washed twice with 500µl of Wash Buffer II. The filter was dried by centrifugation for 1 minute at 10,000 x g and placed in a new collection tube. 30µl of dH2O was added to the filter, incubated at room temperature for 1 minute and centrifuged for 1 minute to collect the purified miRNA. The miRNA was quantitated using a Nanodrop spectrophotometer.

2.18.4.9 Preparation of amplified miRNA for microarray analysis

Approximately 1µg of miRNA was needed for array experiments. The volume of sample containing that amount was calculated from its concentration and aliquoted into a 0.5ml tube and dried using a vacuum centrifuge and stored at -80°C. As the amplified miRNA samples were already polyA-tailed we proceeded directly to the labeling step in section 2.17.3.3.

2.18.5 miRNA array analysis using Agilent system

Arrays were carried out using Agilent Human miRNA microarray slides and the Agilent miRNA labelling and hybridization kit (Agilent Technologies Inc., Santa Clara, CA, USA). Each array slide contains eight microarrays printed on a 1-inch × 3-inch glass slide and has 15,000 probes based on the Sanger mirbase version 14.0.
2.18.5.1 Preparation of spike-in controls

All components of the microRNA Spike-In Kit (Agilent) were thawed and briefly centrifuged to collect the contents. Five tubes were labelled “1st Dilution Labelling Spike-In” and five tubes labelled “1st Dilution Hyb Spike-In” and 198µl of the dilution buffer was added to each of the tubes. 2µl of the labeling Spike-In solution was added to the first five tubes and 2µl of Hyb Spike-In solution added to the second five tubes. The tubes were mixed well and briefly centrifuged. One tube of each dilution was used at a time and the other tubes stored at -80°C.

Four new tubes were labelled for 2nd and 3rd Dilution Labelling Spike-In and 2nd and 3rd Dilution Hyb Spike-In and 198µl of nuclease-free H₂O added to each tube. To make the 2nd Dilution Spike-In solutions, 2µl of 1st Dilution Labeling Spike-In and 1st Dilution Hyb Spike-In were added to the respective 2nd dilution tubes and mixed well. To make the 3rd Dilution Spike-In solutions, 2µl of 2nd Dilution Labeling Spike-In and 2nd Dilution Hyb Spike-In were added to the respective 3rd dilution tubes and mixed well. The 3rd dilutions were the working solutions used in the following reactions and were made up fresh for each array experiment.
2.18.5.2 Dephosphorylation
Total RNA extracted from HB samples (Section 2.5) were used in array experiments and the concentration determined using the Nanodrop spectrophotometer. Each total RNA sample was diluted to 50ng/µl in nuclease-free H₂O and 2µl (100ng) of the diluted RNA transferred to a 0.2ml PCR tube and kept on ice. Immediately prior to use, a master mix was prepared containing Calf Intestinal Alkaline Phosphatase (CIP) and 2µl added to each sample and mixed gently by pipetting. The samples were dephosphorylated by incubation at 37°C in a thermocycler for 30 minutes.

<table>
<thead>
<tr>
<th>10x CIP buffer</th>
<th>0.4µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeling Spike-In</td>
<td>1.1µl</td>
</tr>
<tr>
<td>CIP</td>
<td>0.5µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2µl</strong></td>
</tr>
</tbody>
</table>

2.18.5.3 Denaturation
2.8µl of 100% DMSO was added to each sample and incubated at 100°C for 5 minutes. The samples were immediately transferred to an ice-water bath.

2.18.5.4 Ligation
The 10x T4 RNA Ligase Buffer was warmed at 37°C and vortexed to dissolve the precipitate and then cooled to room temperature. Immediately prior to use, a Ligation Master Mix was prepared containing the components listed below and 4.5µl of this master mix added to each tube and gently mixed by pipetting. The samples were incubated for 2 hours at 16°C. The samples were then fully dried using a vacuum concentrator at 50°C for ~2 hours.

<table>
<thead>
<tr>
<th>10x T4 RNA Ligase Buffer</th>
<th>1µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanine3-pCp</td>
<td>3µl</td>
</tr>
<tr>
<td>T4 RNA Ligase</td>
<td>0.5µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>4.5µl</strong></td>
</tr>
</tbody>
</table>
2.18.5.5 Hybridization preparation
To prepare the 10x Blocking Agent, 125μl of nuclease-free H₂O was added to the vial containing lyophilised 10x GE blocking agent and mixed gently by pipetting. The dried miRNA samples were resuspended in 17μl of nuclease-free H₂O and 1μl of Hyb Spike-In added to each sample. 4.5μl of the 10x Blocking agent and 22.5μl of Hi-RPM Hybridization buffer added to each sample and mixed well. The tubes were incubated at 100°C for 5 minutes and transferred to an ice-water bath for 5 minutes. The samples were centrifuged briefly to collect the sample.

2.18.5.6 Hybridization Assembly
To assemble the array system, a clean gasket slide was loaded into the Agilent SureHyb chamber base with the label facing up and carefully aligned. The entire volume (45μl) of each labeled miRNA sample was pipetted onto one of the eight gasket wells. The array slide was placed “active side” down onto the SureHyb gasket slide so that the “Agilent”-labeled barcode was facing up. The SureHyb chamber cover was placed onto the sandwiched slides and the clamp assembly hand-tightened. The assembled slide chamber was placed in a rotisserie in a hybridization oven set to 55°C for 20 hours.

2.18.5.7 Microarray Wash
To prepare the Gene Expression wash buffers, 2ml of Triton X-102 was added to each wash buffer container, mixed well and labelled. The Gene Expression wash buffer 2 was warmed overnight to 37°C in a hot room. Designated glassware was washed with copious Milli-Q water prior to array washing. Two slide-staining dishes were filled with Gene Expression wash buffer 1 at room temperature and one staining dish filled with Gene Expression wash buffer 2 at 37°C. The hybridization chamber was removed from the incubator and disassembled transferring the array-gasket sandwich quickly to the first dish of Gene Expression wash buffer 1. When completely submerged in buffer, the array sandwich was gently pried open at the barcode end using a forceps. The microarray slide was removed and placed in a slide colder in the second dish of Gene Expression wash buffer 1. A magnetic stirrer was used at a moderate speed for 5 minutes. The slide rack was then transferred to Gene Expression
wash buffer 2 at 37°C for 5 minutes with stirring. The slides were slowly removed from the buffer and scanned immediately.

### 2.18.5.8 Array Scanning and Feature Extraction

An Agilent Scanner Version C was used to scan the miRNA arrays using the AgilentHD_miRNA setting and the Scan Control version 8.0. Table 2.4 shows the scan settings used.

<table>
<thead>
<tr>
<th>Scan Setting</th>
<th>Values for 8x15K Formats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan region</td>
<td>Scan Area (61 x 21.6mm)</td>
</tr>
<tr>
<td>Scan resolution (µm)</td>
<td>5</td>
</tr>
<tr>
<td>5µm scanning mode</td>
<td>Single pass</td>
</tr>
<tr>
<td>eXtended Dynamic range</td>
<td>selected</td>
</tr>
<tr>
<td>Dye channel</td>
<td>Green</td>
</tr>
<tr>
<td>Green PMT</td>
<td>XDR Hi 100%, XDR Lo 5%</td>
</tr>
</tbody>
</table>

Agilent Feature Extraction (FE) version 10.7 was used to measure miRNA expression in the samples from the information generated by the miRNA array. The FE software generates two text files for each 8x15K array. One file includes all features, probe and gene level data, array statistics and can be used to view the quality control (QC). Figure 2.3 shows an example of a QC report generated by the FE version 10.7 software. The second “GeneView” file is a simple file that is appropriate for any further analysis and contains a set of values that has been background subtracted and outlier rejected.
Figure 2.3: A QC Report for 8x15K miRNA microarray, generated by Feature Extraction version 10.7 with the use of Spike-In miRNAs.

2.18.6 Slide stripping procedure for microRNA array slides

Commercially obtained 20x SSC (Ambion) was diluted 1:200 with ddH$_2$O to give a concentration of 0.1 x SSC. The microarray slides were rinsed in milliQ water for 1 minute and immersed in approximately 250ml of pre-warmed 0.1 x SSC stripping buffer in a staining dish. The slides were incubated for 10 minutes at 62°C and the incubation was repeated once with fresh buffer. The slides were then removed and washed in three changes of ddH$_2$O for 15 minutes each time with gentle agitation at room temperature. The slides were then slowly removed from the water bath and excess moisture shaken off. Slides were scanned to verify the effects of the stripping buffer and stored in the dark until reuse.
Chapter Three
Hepatoblastoma Biomarker Study
Chapter 3: Hepatoblastoma biomarker study

3.1 Introduction

3.1.1 Background

Hepatoblastoma (HB) is a very rare childhood liver cancer. The very poor survival rate has improved dramatically in recent years to greater than 70% with the advent of platinum-based neo-adjuvant chemotherapy. However, there still remains about 10-15% of standard risk patients that die of the disease and current risk factors in use such as PRETEXT staging and AFP level do not identify these cases. Recurrence or progression of the disease in patients after treatment is associated with a very low survival rate of around 20%. Additionally, patients identified as high-risk have only a 35% chance of survival from HB.

Currently, risk stratification is carried out purely on the basis of clinical factors. With the exception of serum AFP levels, no biological markers are used either in diagnosis or prognostication. As the tumour is so rare it is impossible for a single centre to amass sufficient cases to evaluate potential biomarkers with respect to classification and outcome. With the advent of clinical trials like those of SIOPEL and COG (Meyers et al., 2009), tumour material is assessed centrally and clinical, histologic and survival data can be accessed more easily. In this study we aim to evaluate potential biomarkers to aid identification of potential relapse cases that would otherwise be overlooked by current prognostication. This may identify patients that would benefit from more aggressive therapy and could improve overall survival rates. Analysis of biomarkers may also be useful in histologic classification of this complex tumour and also in understanding the biological pathways involved in tumorigenesis of HB.

We sought to test the hypothesis that specific proteins implicated in HB biology would be differentially expressed in tumours of relapse compared to non-relapse patients. We also sought to determine whether these proteins could differentiate between histologic subtypes. Five biomarkers, β-catenin, E-cadherin, CyclinD1, Ki-67 and AFP were chosen for analysis in our cohort based on a review of the literature
published about HB. The study also aims to verify results previously published in small studies.

SIOPEN 3 patients are located in many different centres worldwide and access to tissue blocks was at the discretion of the individual centre. As the tumour is so rare and tumour material had to be returned to the participating centres it was decided to construct a Tissue Microarray (TMA) using 1mm cores of tissue from the area of interest of each tumour. This allows for the creation of multiple (<100) 4µm serial sections for analysis of multiple potential markers of disease.

3.1.2 Tissue Microarrays
The major limitations in molecular analysis of clinical tissues include the cumbersome nature of procedures, limited availability of diagnostic reagents and limited patient sample size. The technique of tissue microarray was developed to address these issues. Before the advent of TMAs, a pathologist placed individual tissue sections by hand on separate glass slides for further staining and analysis. A tissue microarrayer essentially automates this process. The instrument obtains cylindrical specimens (tissue cores) from standard histological paraffin blocks and arranges them into a single paraffin block in a precise array pattern. Prepared sections of the TMA block (typically 4 to 5 microns thick) containing the arrayed tissue are placed on histological glass slides for further microscopic examination.

Multi-tissue-blocks were first introduced by Battifora in 1986, with his so called “multitumor (sausage) tissue block” and modified in 1990 with its improvement, "the checkerboard tissue block” (Battifora, 1986, Battifora and Mehta, 1990). In 1998 Kononen et al developed the current technique, which uses a novel sampling approach to produce tissues of regular size and shape that can be more densely and precisely arrayed (Kononen et al., 1998).

3.1.3 Advantages of tissue microarrays over regular sections
Tissue microarrays have some very obvious advantages over work with standard tissue sections. Speed and increased throughput are due to the large number of samples present on a single slide. Special staining techniques and
immunohistochemistry can be quite laborious when using single tissue sections. TMAs are easy to use once created and also allow for standardization of analyses, as inter-batch variation is common in the techniques used. Paraffin-embedded tissue is a finite resource and TMA construction is a means of conservation of valuable tissue as only small cores are used. TMAs are also compatibility with various image-analysis techniques, including automated and remote pathology diagnosis.

Figure 3.1: Schematic diagram showing the construction of a tissue microarray (Giltnane and Rimm, 2004)
3.1.4 Applications of tissue microarrays in cancer research

To date immunohistochemistry and fluorescent in situ hybridization (FISH) techniques have been used on tissue microarrays to analyze the frequency of a molecular alteration in different tumour types (Chen et al., 2007, Hwang et al., 2007). TMAs have also been used for evaluation of prognostic markers and testing the power of potential diagnostic markers.

Figure 3.2: Example of a tissue microarray section used in this study. A: It contains 120 hepatoblastoma specimens stained with an antibody to β-catenin. B: Four magnified (10x) cores of HB from the boxed area in (A) C: A further magnification (40x) of the boxed area in (B).
3.1.5 Limitations of TMAs
The amount of tissue required for a representative analysis has been an area of debate in the use of TMAs. Camp et al have shown that as little as two cores give >95% accuracy compared with an entire tissue section and similar results have been found in other validation studies (Camp et al., 2000, Mucci et al., 2000). As HB is histologically heterogeneous, a single 1mm tissue core may not always reflect the biological property of the entire tumour. To overcome this, a duplicate TMA block was constructed using cores from distinct representative areas of the tumour, and sections from both arrays were analyzed together.

3.2 SIOPEL Hepatoblastoma tissue array
3.2.1 Array design
Our HB-TMAs consisted of 116 samples in two blocks named 1A and 2A, and the remaining 42 samples in Blocks 1B and 2B. Each block also had 4 control cores (two normal kidney and two breast cancer) that were used to orientate the array. Of 158 sample cores, 111 were tumour tissue and 47 were from adjacent normal tissue. Both diagnostic and post-chemotherapy samples were available in 17 cases. An excel spreadsheet was used to identify each sample core position in the TMA (See Figure 3.3 and 3.4 below)

Figure 3.3: Excel sheet layout of TMA-1A
3.2.2 Histologic features of the HB cohort

In this study we have accessed formalin-fixed paraffin-embedded tumour samples from 91 HB patients. All samples were from patients registered on the SIOPEL-3 trial, which makes our cohort uniquely homogenous. The cohort consists of 41 diagnostic biopsies and 69 samples from post-chemotherapy tumours. Where both pre- and post-chemotherapy specimens were available for 19 patients, the diagnostic biopsy was used in the analysis. Histologic information was available for all but three patients. The tumours were all examined centrally and classified as either wholly epithelial (n=57) or mixed epithelial and mesenchymal (n=28). One tumour was diagnosed as hepatocellular carcinoma, one as fibrolamellar type and one as a transitional liver cell tumour. The epithelial component was further subtyped as pure fetal (n=38), embryonal (n=2) or mixed fetal and embryonal (n=28). Two tumours were subtyped as macrotrabecular type A, three had areas of macrotrabecular B and four were classified as small cell undifferentiated tumours (one of these having only small foci of SCUD). Focal anaplasia was seen in three tumours and cholangioblastic features in two tumours. Thirteen cases of osteoid formation were noted in the histology reports with additional osteoid formation in a post-chemotherapy sample that lacked osteoid in the diagnostic biopsy. Teratoid features were noted in seven cases.

Figure 3.4: Excel sheet layout of TMA-1B.
3.2.3 Clinical characteristics of patients for survival analysis

For the purposes of survival analysis only the 71 patients that were classified into the two well-defined risk groups were included. Twenty-seven of these were high-risk and forty-four were standard risk. Of these 71 patients, nine were born with low birth weight. PRETEXT classification revealed that there were two PRETEXT stage 1 patients, twenty-two stage 2, thirty-one stage 3 and sixteen stage 4 patients. Only two patients had serum AFP levels of <100 at diagnosis, making them high-risk. Eight and seven patients had portal vein and vena cava involvement respectively, and extrahepatic intra-abdominal disease was seen in three patients also making them high-risk cases. Metastatic disease was present at diagnosis in thirteen children. Relapse or progression in five HR cases resulted in the death of four patients. In the standard-risk group there were six relapses leading to a single death due to disease (See Appendix Table 1 for comprehensive histologic and clinical data on our cohort).

3.3 Results

3.3.1 Variation in sample numbers for each biomarker

One of the limitations of working with TMAs is the difficulty of cutting sections containing the total number of cores. Due to different histologic components and variation in fixation of the tumour samples, some cores cut differently to others. This can cause them to curl up during sectioning. Other cores float away as they are being placed on the slide or during the antigen-retrieval process. Also as the tumour thickness from the original blocks varies, some cores are cut through before others. All of these issues mean that the number of tissue cores on each slide varies and give rise to varying numbers of samples in the biomarker analysis.

3.3.2 Investigation of β-catenin protein expression in HB

β-Catenin is the critical mediator of the canonical Wnt pathway. The Wnt/β-catenin pathway involves proteins that are directly involved with both gene expression and cell adhesion. Its role in normal liver biology includes embryonic development, hepatoblast differentiation, hepatocyte differentiation and xenobiotic metabolism (Monga, 2009). Aberrant Wnt/β-catenin has been implicated in many cancers and has been identified as an important pathway in both HB and adult HCC (Peifer and
Polakis, 2000). Ihara et al first demonstrated the abnormal accumulation of catenins in liver cancer by IHC and subsequent studies have shown abnormal β-catenin in up to 90% of HCC and 100% of HBs (Ihara et al., 1996, Laurent-Puig et al., 2001). Thus the accumulation of β-catenin appears to be a crucial event in the tumorigenesis of HB although the level of protein accumulation varies between 67% and 100% in reported studies.

3.3.2.1 Results of IHC analysis of β-catenin
We examined β-catenin expression in our HB cohort of 158 samples from 94 patients using IHC. Results were available for 147/158 samples. In eleven cases the tissue cores had lifted during section cutting or staining. Adjacent normal tissue showed only membranous staining as shown in Figure 3.5.

**Figure 3.5:** Staining of normal adjacent liver tissue using an antibody to β-catenin. Nuclei are stained blue with a haematoxylin counterstain and membranous staining of β-catenin is brown.
Staining results were available for 102 tumour samples comprising nine samples (9%) with a normal membranous pattern, twelve with no staining (11%) and the remainder (80%) with some type of aberrant protein accumulation. Abnormal staining was deemed to be focal or diffuse in the cytoplasm and/or nucleus and various permutations of staining pattern occurred. Forty-eight samples (48%) displayed staining in the cytoplasm alone (Figure 3.6), 24 samples (23%) showed both nuclear and cytoplasmic staining (Figure 3.7) and 9 samples (9%) had solely nuclear staining using an antibody to β-catenin.

Figure 3.6: Cytoplasmic localization of β-catenin. Cytoplasmic staining of HB cells using an antibody to β-catenin in brown with nuclear counterstaining in blue at a magnification of 40x.

The staining intensity varied between sample cores but this was not taken into account when scoring the IHC for β-catenin as tissue fixation and storage have been shown to impact greatly on this. Section 2.4.11 details the scoring used for analysis of β-catenin. Concurrent membranous and abnormal staining patterns were seen in some tumour cases, which may reflect different cell populations within the tumour. Our
results reflect a previous study that reported a higher incidence of nuclear β-catenin in embryonal and SCUD type HB cells although this is not reflected statistically (Park et al., 2001b).

Figure 3.7: Nuclear localization of β-catenin. Strong nuclear positivity in addition to cytoplasmic staining using an antibody to β-catenin in HB shown in brown with haematoxylin counterstain in blue.

3.3.2.2 Statistical Analysis of β-catenin expression in our cohort

Statistical analysis was carried out to correlate β-catenin expression with histologic type. For the analysis only results from tumour samples were used and biopsy (pre-chemotherapy) specimen results were used where both pre- and post-chemotherapy samples were available. This was to minimise the impact of chemotherapy-treated samples on the interpretation of the data. In seven cases, no biopsies were available so post-chemotherapy tumour samples were used. Three patients were excluded from the analysis; one lacked any tumour tissue, one was categorized as transitional liver cell tumour and another as adult type HCC. Frequency tables were generated to create an overview of patients in each histologic subtype. Even with our large cohort, the wide range of histologic subtypes meant that there were small numbers in some groups.
For statistical purposes only histologic types with greater than ten patients were taken into account and smaller categories were omitted. Fisher’s exact test was used to generate correlations and a 2-tailed observed $P$-value less than 0.05 indicated a significant correlation.

IHC results were grouped into abnormal (any cytoplasmic or nuclear staining) or other (normal membranous or negative). No statistical correlation was observed between IHC results for $\beta$-catenin and any histologic type or subtype in HB ($P=0.569$). Survival analysis was then carried out to ascertain whether $\beta$-catenin was of potential prognostic use as a biomarker. Within our cohort there were six patients with events; relapse or death of disease. The Lifetest procedure was used to test homogeneity of survival curves for event-free survival and overall survival (OS) over strata. Chi-square analysis showed no significant relationship between $\beta$-catenin status and EFS or OS (Figure 3.8).

![Figure 3.8: Survival curves for $\beta$-catenin protein expression.](image)

Our results show that $\beta$-catenin as a biomarker does not add any information to prognostication or histologic classification in HB. The existence of different staining patterns of $\beta$-catenin within tumours may mark different hepatic cell lineages but
would not benefit reporting of this rare tumour. However the high expression of \(\beta\)-catenin in tumour samples reflects that published previously and is consistent with the known role of \(\beta\)-catenin in the pathogenesis of HB. Therefore further investigation into the role and mechanism of \(\beta\)-catenin in HB is merited and may lead to a better understanding of the disease.

### 3.3.3 E-Cadherin as a biomarker for HB

E-Cadherin is the major cell-cell adhesion molecule in epithelial cells, including liver cells. Epithelial cells are tightly interconnected through tight junctions, adherens junctions (AJ) and desmosomes. In the case of AJs, \(\beta\)-catenin is bound to the cytoplasmic domain of E-cadherin at the cell membrane and provides the link between the cadherin and the cytoskeleton (Kemler, 1993). E-cadherin acts as a tumour suppressor as loss of expression or a switch from E-cadherin to N-cadherin is the hallmark of epithelial-mesenchymal transition (EMT) (Wu and Zhou, 2008). As well as a vital process during embryonic development, organogenesis and tissue remodelling, EMT is thought to be the mechanism by which many cancers metastasize (Weinberg, 2008a). Decreased levels of E-cadherin correlate with dedifferentiation, infiltration and metastases (Semb and Christofori, 1998, Christofori and Semb, 1999). E-Cadherin function has shown to be reduced during carcinogenesis in breast, colon, prostate, lung, stomach and liver and expression of the protein has prognostic implications in gastric and thyroid cancers (Bohm et al., 1994, Furuta et al., 2006, Gabbert et al., 1996, Ghadimi et al., 1999, Scheumman et al., 1995, Zschiesche et al., 1997). As HB is an epithelial cancer with embryonic origins it is very likely that mis-regulated EMT is involved in its pathogenesis and E-cadherin expression could be a useful biomarker.

#### 3.3.3.1 Results of immunostaining of E-cadherin

IHC staining results were available for 102 of 113 HB samples using an antibody to E-cadherin. All normal samples showed a membranous staining pattern (figure 3.9) and were not included in any statistical analysis. In 64 cases (63%), the tumour cells showed a normal membranous staining pattern. 32 samples (31%) showed a heterogeneous staining pattern with different tumour elements within a sample core.
showing normal staining or lacking E-cadherin expression (Figure 3.10). Only six samples (6%) showed complete loss of E-cadherin expression.

**Figure 3.9: Normal E-cadherin staining pattern.** Normal tissue adjacent to HB stained with an antibody to E-cadherin. The normal membranous staining pattern in brown with nuclear counterstain in blue.

**3.3.3.2 Statistical analysis of E-cadherin expression**

Statistical analysis was carried out as detailed above. E-cadherin status was deemed normal or else (heterogenous or negative). No correlation was found between E-cadherin expression and histologic type or subtype ($P=0.154$). Survival analysis did not show E-cadherin as a statistically significant predictor of outcome in our cohort with a $P$-value of 0.332 (Figure 3.11). The heterogeneous staining pattern seen in many tumour samples may reflect different progenitor cell origin and loss of expression in subpopulations may indicate more aggressive tumour cells capable of metastasis. Although its use as a clinical marker is limited, study of E-cadherin expression patterns in different cell types could aid with the identification of metastatic cells in HB.
Figure 3.10: Heterogenous E-cadherin expression. The insert shows an area of HB tumour with normal membranous staining (in brown) and surrounding tumour where expression is lost. Nuclei are stained blue with a haematoxylin counterstain.

Figure 3.11: Survival curves for E-cadherin expression. Positive E-cadherin expression shown in red and negative in black ($P=0.332$).
3.3.4 Alpha-fetoprotein as a potential biomarker in HB

Alpha-fetoprotein (AFP) is thought to be the foetal counterpart of serum albumin and the alpha-fetoprotein and albumin genes are present in tandem on chromosome 4. In humans, AFP levels decrease gradually after birth, reaching adult levels by 8 to 12 months. Normal adult AFP levels are low, but detectable, although AFP has no known function in healthy adults. In normal foetuses, AFP binds the hormone estradiol. It is measured in pregnant women, other adults, and children, serving as a biomarker to detect a subset of tumours, principally hepatocellular carcinoma (HCC). Although AFP is normally high in infancy, the level is exceptionally elevated in children with HB.

Patients with normal or low levels of AFP and those with extremely high levels have a poor prognosis, in contrast to those with intermediate values (von Schweinitz et al., 1997b). In one study, patients with low levels had the small-cell undifferentiated (SCUD) variant, which grows rapidly, and usually does not respond to chemotherapy. Extremely high levels of AFP were associated with extensive and/or metastatic tumours, and thus an unfavourable outcome (De Ioris et al., 2008). AFP also is used to monitor response to therapy and to detect tumour recurrence after treatment. In one study of 31 children with primary unresectable HB, a large, early fall in AFP level in response to chemotherapy was the strongest independent predictor of outcome (Van Tornout et al., 1997). Patients with recurrent disease may demonstrate an elevated AFP level long before observing imaging evidence of tumour recurrence (Kubota et al., 2004). We examined AFP protein expression in our tumour cohort using IHC to explore its prognostic value as a biomarker, and also to look for a correlation between serum AFP and protein expression in the tumour.

3.3.4.1 Results of IHC staining of AFP

Immunostaining results were available for 83 samples of HB using an antibody to alpha-fetoprotein. Pathologist examination designated staining as negative or cytoplasmic (focal or diffuse). No staining of nuclei was seen. Figure 3.13 shows negative staining reported in 71/83 samples (86%). Twelve tumour samples had cytoplasmic expression of AFP, seven with focal staining (8%) and five with diffuse cytoplasmic staining (6%) (Figure 3.12).
**Figure 3.12: Positive AFP staining.** Staining with an antibody to AFP in HB shows positive nuclei staining brown against a haematoxylin counterstain.

### 3.3.4.2 Statistical analysis of AFP expression in HB

For statistical purposes AFP expression was reported as positive or negative. No correlation was found with histologic type or subtype ($P=0.51$). Neither was AFP protein expression a predictor of outcome in survival analysis (Figure 3.14). Analysis was carried out to determine if the serum level of AFP at diagnosis correlated with the expression of the AFP protein in the resected tumours. No statistically significant correlation was found ($P=0.34$).
Figure 3.13: Negative staining for AFP in hepatoblastoma. Nuclei stain with the blue haematoxylin and show no brown staining.

Figure 3.14: Survival curve for AFP. AFP positive tumours in red and AFP negative in black ($P=0.32$).
3.3.5 Cyclin D1 as a biomarker in HB

Cyclin D1 is a critical cell-cycle mediator and a transcription target of β-catenin. The mechanism by which it promotes cancer progression is still a matter of intense study but several studies have shown that overexpression occurs even at early stages of carcinogenesis and also in precancerous lesions (Alao, 2007). Amplification or overexpression of Cyclin D1 is seen in many cancers including parathyroid adenoma, breast, prostate and colon cancers, lymphoma and melanoma but the prognostic value is still a matter of debate (Liao et al., 2007). Cyclin D1 expression is regulated by β-catenin and as β-catenin plays a crucial role in HB, Cyclin D1 may be useful as a biomarker of disease progression.

Figure 3.15: Staining of Cyclin D1 by IHC shows a heterologous staining pattern of positive (brown) and negative (blue) nuclei.
3.3.5.1 Results of immunostaining with an antibody to Cyclin D1

IHC results were available for 104 tumour samples using an antibody to Cyclin D1. These were categorized as negative or positive. Positive staining was seen only in the nucleus in 35/104 (34%) of cases (Figure 3.15) and the remaining tumours were negative (Figure 3.16).

Figure 3.16: Cyclin D1 negative staining. Hepatoblastoma negative for staining with an antibody to Cyclin D1. Nuclei are counterstained with blue haematoxylin and show no brown staining.
3.3.5.2 Statistical analysis of Cyclin D1 staining in HB

Statistical analysis was carried out for CyclinD1 as for biomarkers discussed previously. Fisher’s exact test showed a correlation between histologic subtype at biopsy and Cyclin D1 protein expression in tumours. The analysis showed that mixed epithelial/mesenchymal type HB are more likely to have Cyclin D1 expressed in the nuclei of tumour cells than the pure foetal subtype. The $P$-value was 0.07, which indicates that the results are approaching significance and possibly in a larger cohort would be statistically significant. In addition to this interesting finding, survival analysis revealed that Cyclin D1 expression correlates with EFS in our HB cohort ($P=0.01$). This shows that Cyclin D1 may predict outcome and could potentially be used as a biomarker for prognostication in HB (Figure 3.17).

![Cyclin-D1 and EFS](image)

**Figure 3.17: Survival curves for Cyclin D1.** The black curve represents patients negative for Cyclin D1 expression and the red curve shows patients that are Cyclin D1 positive.

3.3.6 Ki-67 as a biomarker for HB

The Ki-67 protein is a cellular marker for proliferation. During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis, most of the protein is relocated to the surface of the chromosomes. Ki-67 protein is present during all active phases of the cell cycle ($G_1$, $S$, $G_2$, and mitosis), but is absent from
resting cells \( (G_0) \). Ki-67 is an excellent marker to determine the growth fraction of a given cell population. The fraction of Ki-67-positive tumour cells (the *Ki-67 labelling index*) is often correlated with the clinical course of cancer.

The best-studied examples in this context are carcinomas of the prostrate and the breast. For these types of tumours, the prognostic value for survival and tumour recurrence has repeatedly been proven in uni- and multivariate analysis (Stuart-Harris et al., 2008, Roach et al., 2009). A study involving patients with HCC showed nuclear beta-catenin staining correlated significantly with increased Ki-67 proliferative index and seemed to be associated with poor outcome (Nhieu et al., 1999). A small study in HB examined Ki-67 staining using the MIB-1 antibody and found a correlation between clinical stage and poor outcome with Ki-67 expression (Ara et al., 1997). We sought to validate these results in a large cohort of HB patients and to test for correlations with histologic subtype.

### 3.3.6.1 Results of Immunohistochemistry using an antibody to Ki-67

Staining Ki-67 protein with the MIB-1 antibody was successful in 104 tissue cores. Of these 8 samples were completely negative and the positive nuclear staining was reported as a percentage of tumour nuclei counted (Figures 3.18 and 3.19). The range of staining was from 0% to 75% with the median being 9%.

### 3.3.6.2 Statistical Analysis of Ki-67 staining results

Statistical analysis of Ki-67 expression was carried out in our cohort as described previously. The results were divided into <10% nuclear staining and >10% nuclear staining for the purposes of our analysis. No correlation was found between histologic subtype and percentage of Ki-67 staining \( (P = 0.683) \). Survival analysis shows that a high Ki-67 positivity correlated with outcome (recurrence/death) with a \( P \)-value of 0.011 using the log-rank test (Figure 3.20). This result corroborates the earlier results of Ara et al., and reflects the findings of many cancer studies (Ara et al., 1997).
Figure 3.18: High Ki67 score. IHC showing strong nuclear staining with an anti-Ki67 antibody in a population of hepatoblastoma cells.

Figure 3.19: Low Ki67 score. A case of hepatoblastoma with a low Ki67 score indicated by brown staining nuclei.
3.4 Discussion

This study aimed to evaluate the histologic and prognostic value of various biomarkers in a large cohort of HB patients. The outcome for HB patients has dramatically improved in recent times with the use of Platinum-based chemotherapy and clinical trials have identified factors such as PRETEXT staging and AFP levels that predict outcome. However, even using these prognostic indicators, around 10% of standard risk patients relapse or die of disease. Identification of a biomarker that could identify this small subset and mark them for more aggressive chemotherapy would be beneficial in the clinical setting. The histology of HB is very complex and involves multiple subtypes that are constantly being revised and updated. A biomarker that could differentiate between histologic subtypes would be useful in the treatment of HB as one type, pure fetal HB, may not need any chemotherapy whereas SCUD HB requires an aggressive approach to treatment. Previous IHC studies of HB have proposed the use of certain markers but none were large enough to have statistical power.
Our study has identified one biomarker, Cyclin D1, which has a correlation with mixed epithelial/mesenchymal HB approaching significance ($P = 0.07$). The other four markers we examined have no correlation with histology. This may reflect the complex classification and the many different types of tumour cells found within a single tumour sample. While our results do not aid classification, they may help to unravel the varied mechanisms underlying the pathogenesis of this complex tumour.

Survival analysis using these markers has revealed two potential prognostic indicators; Cyclin D1 and Ki-67. Ki-67 was previously proposed as a predictor of outcome and we have successfully validated that result in our larger cohort. Evaluating the expression of these two proteins by IHC in HB samples could identify standard-risk patients that should be stratified as high risk and treated accordingly. Due to the low number of events in our cohort, analysis of Cyclin D1 and Ki-67 expression in a larger group of patients enriched for relapse/death would be necessary to validate our findings prior to use in a clinical trial setting.

In conclusion, the construction of a hepatoblastoma tissue-microarray and its use in a biomarker study has both prognostic use and potential as a tool for translational research in HB. Future discoveries depend on the acquisition of larger numbers of tumour samples from the SIOPEL trials to validate our findings.
Chapter Four

Activation of β-catenin by c-Met

in Hepatoblastoma
Chapter 4: Activation of β-catenin by c-Met in hepatoblastoma

4.1 Introduction

Despite accounting for 1% of all childhood malignancies, hepatoblastoma (HB) is the most common liver cancer in children (Weinberg and Finegold, 1983). Most cases are sporadic although some are associated with Beckwith-Weidemann syndrome or familial adenomatous polyposis (DeBaun and Tucker, 1998, Oda et al., 1996). HB has a very complex histology with subtypes ranging from the aggressive small-cell undifferentiated type to the purely fetal type of epithelial HB, which has a good prognosis. In addition to fetal and embryonal epithelial types, mixed epithelial/mesenchymal HBs contain mesenchymal elements such as chondroid and osteoid (Stocker, 1994). The varied histopathology reflects distinct patterns of embryonal liver development and maturation, and indicates a developmental origin for HB.

Of several distinct developmentally regulated pathways known to be active in hepatoblastoma, such as IGF2/H19, Shh/PTCH, Notch, and Wnt/β-catenin, it is the Wnt/β-catenin pathway that is most closely implicated in the origins of this tumour (Koesters and von Knebel Doeberitz, 2003). Previous tissue based studies using immunohistochemistry show nearly all hepatoblastomas have aberrant accumulation of β-catenin protein (Koch et al., 1999, Jeng et al., 2000, Yamaoka et al., 2006).

Several studies of sporadic HB have also identified mutations in CTNNB1, the gene coding for β-catenin, suggesting it has a crucial role in tumorigenesis (Wei et al., 2000, Udatsu et al., 2001, Blaker et al., 1999, Buendia, 2002). Canonical Wnt/β-catenin signalling is known to be a key regulator of cell proliferation, differentiation, zonation and apoptosis during normal liver development (Apte et al., 2007, Nejak-Bowen and Monga, 2008).

Several Wnt/β-catenin related proteins directly alter both gene expression and cell adhesion, with β-catenin protein acting as a critical mediator (Polakis, 2000, Koesters and von Knebel Doeberitz, 2003). In the absence of Wnt signalling, β-catenin is phosphorylated by GSK3β and CK1 at specific serine and threonine residues resulting
in its ubiquitination and subsequent degradation, thus maintaining tight control of β-catenin levels within normal cells (Aberle et al., 1997). Wnt-related activation of the Frizzled receptor, inactivates the APC/Axin/GSK3β degradation complex, inhibiting serine/threonine phosphorylation of β-catenin leading to its accumulation in the cytoplasm. Hypophosphorylated β-catenin binds TCF/LEF transcription factors, translocates to the nucleus and activates expression of target genes, including those involved in cell proliferation (e.g. c-myc and cyclin D1), anti-apoptosis (e.g. survivin), invasion (e.g. matrix metalloproteinases) and angiogenesis (e.g. VEGF) (Cadigan and Nusse, 1997b, Nelson and Nusse, 2004).

Within the cytoplasmic compartment a pool of β-catenin is located at the inner surface of the cell membrane associated with c-Met, the tyrosine kinase receptor for hepatocyte growth factor (HGF, scatter factor) (Monga et al., 2002). Upon signalling by HGF, c-Met protein is activated by tyrosine phosphorylation, which in turn phosphorylates β-catenin at residues Y654 and Y670. This c-Met related tyrosine phosphorylation of β-catenin causes its dissociation from c-Met at the cell membrane, and it blocks the association of β-catenin with axin and GSK3β. This c-Met related β-catenin is not serine/threonine phosphorylated, is not marked for degradation resulting in nuclear accumulation of β-catenin and TCF related gene expression (Figure 1.3). Thus HGF/c-Met related activation of β-catenin occurs independently of the canonical Wnt/β-catenin pathway (Danilkovitch-Miagkova et al., 2001). Pathologic activation of β-catenin by HGF/c-Met signalling is seen in several tumours and is associated with their transformation and its activation has been previously reported in HB (Ranganathan et al., 2005a, Maulik et al., 2002b).

In the previous chapter we found that although β-catenin expression did not correlate with outcome or histologic subtype in our cohort, it was aberrantly expressed in a significant number (89%) of tumour samples. This implies an important role for activated β-catenin in the pathogenesis of hepatoblastoma. In this chapter we investigate the concomitant contribution of Wnt-dependent and Wnt–independent activation of β-catenin in a large homogenous cohort of HB patients.
4.2 Results

4.2.1 HB tumour samples
Our cohort comprises HB patients from the SIOPEL 3 clinical trial who were randomized to receive either cisplatin or a combination of cisplatin and doxorubicin preoperatively. Samples from 98 formalin-fixed paraffin-embedded HB cases were accessed for this study with clinical, histologic and survival data available for most tumours (See Chapter 3). Both diagnostic biopsies and post-resection samples were available in thirty cases. A single 4µm section was cut from each paraffin block and the slides stained with haematoxylin and eosin (H+E). Each slide was examined by a pathologist (Dr. Carina Miles), to select representative tumour and adjacent normal tissue (present in 48 cases) for coring.

4.2.2 High frequency of aberrant β-catenin expression in HB tumours
We examined intracellular β-catenin expression using IHC. Immunostaining for β-catenin was scored as normal membranous, diffuse or focal cytoplasmic and diffuse or focal nuclear staining. Each array duplicate was also stained and the results collated. The staining intensity was noted but not factored, as differing age of donor blocks and variation in fixation methods can impact on staining intensity. The IHC results were analysed in conjunction with two pathologists (Dr Carina Miles and Dr Clinton Turner).

A total of 87% (85/98) of our cohort showed aberrant expression of β-catenin in the cytoplasm and/or nucleus (Figures 3.5 and 3.6). These results are similar to those published previously in HB studies (Park et al., 2001a, Taniguchi et al., 2002, Wei et al., 2000). Normal membranous staining was also observed in some tumour samples in addition to cytoplasmic staining. Samples of adjacent normal tissue had a normal β-catenin staining pattern in most cases (Figure 3.7).

As mentioned in the previous chapter, we found no correlation between β-catenin expression and histologic subtype or outcome ($P = 0.568$ and $P = 0.189$ respectively). However, the frequency of mutations in the $CTNNB1$ gene varies widely in studies of HB, from 13% to 70% (Park et al., 2001b, Wei et al., 2000). To determine whether aberrant β-catenin protein expression may result from an activating mutation in the
gene, we identified the frequency and type of CTNNB1 mutations in our HB cohort. To achieve this we amplified and sequenced the region of CTNNB1 that codes for the GSK3β-binding region in our samples as outlined below.

4.2.3 CTNNB1 mutation detection
Representative areas of tumour and histologically normal tissue were identified on H+E slides by pathologists and a 1mm tissue core removed from corresponding areas on paraffin blocks. The RNA was extracted using RecoverALL™ Total Nucleic Acid Isolation kit (Ambion) as per manufacturer’s instructions. Total RNA was extracted from 124 samples of FFPE hepatoblastoma tumour tissue.

4.2.3.1 Quality control
Samples with the following quality parameters were analysed for CTNNB1 gene mutations: Optical density ratio 260/280 of 1.8 – 2.2 and RNA concentration of >20ng/ul using a Nanodrop spectrometer (Figure 4.1 A). In twenty-one cases the RNA yield and quality was not sufficient for amplification using RT-PCR (Figure 4.1 B). In addition, tissue processing, age of tissue and inadequate storage conditions can all lead to excessive RNA fragmentation so we attempted to amplified a 150pb region of the GAPDH housekeeping gene by RT-PCR to ensure adequate coverage in our samples. Amplification of GAPDH was unsuccessful in the twenty-one samples with poor RNA quality and in an additional three cases. This identified 98 HB samples suitable for sequence analysis.
Figure 4.1: RNA quality measurements. A: Absorbance and concentration of RNA from sample 36bT using the Nanodrop spectrophotometer B: Absorbance and concentration of RNA from sample 35bT using the Nanodrop spectrophotometer. Both the concentration and 260/280 ratio are outside the quality control limits.

4.2.3.2 Amplification of CTNNBI

Primers were designed flanking a 149 bp region of the β-catenin regulatory region of exon 3 of the CTNNBI gene (codons 32-45). Amplification of this region by RT-PCR was successful in 92 of the 98 samples. Lack of amplification in six samples may be due to complete deletion of exon 3 of CTNNBI or fragmentation of the RNA (Figure 4.2). We also attempted to amplify a region spanning exon 2 to exon 4 in these six samples but were unsuccessful. Although not definitive, it is possible that these six samples have deletions in CTNNBI. The samples were then “cleaned up” and
sequenced using both forward and reverse primers. Analysis of the sequence reads was carried out using ABI Variant Reporter v1.0 software and showed good quality traces spanning the region of interest (Figure 4.3). It would also have been possible to extract DNA from the tumour samples and amplification using PCR may have been more successful. However, as starting material was limited and the miRNA fraction could be isolated from RNA for later use, it was decided to use RNA throughout the project.

Figure 4.2: Agarose gel showing amplified β-catenin RT-PCR products. A 100bp DNA ladder are seen in lanes 1 and 8. The positive control is commercially acquired fetal liver RNA, the negative control contains ddH2O and no RNA. Sample 62T did not amplify. The remaining lanes contain β-catenin amplicons from FFPE hepatoblastoma samples 64b, 64a, 63, 62 and 61. Amplification of β-catenin failed in sample 62.
Figure 4.3: Amplicons from samples 82 and 15 with point mutations in forward and reverse sequences highlighted in yellow. These snippets of the CTNNB1 gene amplified from hepatoblastoma samples were generated using Variant Reporter™ software, used to identify variants from the reference sequence.

4.2.3.3 Low frequency of CTNNB1 mutations in our HB cohort

We identified eleven variants of CTNNB1 in 14 of 98 samples (15%). These variants are all point mutations affecting phosphorylation sites in the regulatory region of the protein and have been previously reported (Blaker et al., 1999, Buendia, 2002). The mutations found resulted in the following changes at the protein level; 32D>N, 32D>Y, 32D>V, 32D>A, 33S>P, 33S>C, 34G>R, 34G>E, 34G>V, 35I>P, 35I>S, 37S>Y. One HB patient (CCRG 64) showed the same sequence variation (missense 32D>V) in both diagnostic and post chemotherapy tumour samples. All other pairs of pre- and post-chemotherapy samples were wild type.

Where available, RNA from adjacent normal tissue was also analysed. All of these normal samples displayed wild type CTNNB1, showing that the mutations found were somatic variants. Surprisingly, the frequency of CTNNB1 mutations (14/98) and possible deletions (6/98) in our cohort is lower than the expression of β-catenin protein would lead us to expect. The low number of mutations found and the fact that
all tumours harbouring mutations/deletions displayed aberrant β-catenin expression meant that informative statistical analysis could not be carried out. This prompted us to investigate alternative pathways of β-catenin activation in our tumour cohort.

4.2.4 High levels of HGF/c-Met activation of β-catenin in HB
To investigate the possibility of Wnt-independent activation of β-catenin we stained the hepatoblastoma tissue array, containing 111 tumour samples, with an antibody recognising tyrosine 654-phosphorylated β-catenin (Y654-β-catenin). Tyrosine-654 of the β-catenin protein is phosphorylated by c-Met, the tyrosine kinase receptor for HGF. This phosphorylation activates β-catenin signaling. Staining for Y654-β-catenin was scored as negative, cytoplasmic and/or nuclear staining. This identified whether HGF/c-Met activation of β-catenin is present in this large subset of HB samples. In total 52 samples had cytoplasmic staining with the antibody (Figure 4.4 B). 30 samples showed nuclear accumulation in addition to cytoplasmic staining (Figure 4.4 C) while 16 samples were completely negative for Y654-β-catenin (Figure 4.4 A).

4.2.4.1 Correlation between tyrosine phosphorylated β-catenin and CTNNB1 mutation status
We examined the correlation between mutation status of the CTNNB1 gene and the expression of the Y654-β-catenin protein in hepatoblastoma. Figure 4.5 depicts the frequencies of total β-catenin and Y654-β-catenin expression in wild type and mutated hepatoblastomas studied. IHC analysis showed that 5/20 (25%) cases harbouring mutations/deletions were completely negative using the Y654-β-catenin antibody, 14/20 (70%) had cytoplasmic staining alone, and only one of 20 (5%) had nuclear expression in addition to cytoplasmic staining. In contrast, 27/78 (34%) of HB cases with wild type CTNNB1 showed nuclear localization of Y654-β-catenin, 44/78 (57%) showed cytoplasmic staining only and 7/78 (9%) were negative for staining.

Statistical analysis was carried out by Dr. John Pearson using the R statistical analysis package (R Development Core Team, 2009). This revealed a significant correlation between nuclear accumulation of tyrosine 654-phosphorylated β-catenin and HB tumours lacking a CTNNB1 mutation in exon 3 (P-value = 0.015). Correlation of Y654-β-catenin expression with histologic type and subtype did not reveal any
significant associations \((P=1, P=0.093\) respectively). No correlation was found between Y654-\(\beta\)-catenin and clinical outcome either \((P=0.92)\).

Table 4.1. Histologic type and subtype, \(\beta\)-catenin and Y654 \(\beta\)-catenin IHC and CTNNB1 gene status of hepatoblastomas with mutations.

<table>
<thead>
<tr>
<th>Case Number</th>
<th>Histologic Type</th>
<th>Histologic Subtype</th>
<th>(\beta)-catenin</th>
<th>Y654-(\beta)-catenin</th>
<th>CTNNB1 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRG9</td>
<td>Epithelial</td>
<td>Pure fetal</td>
<td>dc</td>
<td>cyto</td>
<td>32D&gt;Y</td>
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<td>Fetal /embryonal</td>
<td>n/r</td>
<td>neg</td>
<td>33S&gt;C</td>
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<td>CCRG16</td>
<td>Mixed</td>
<td>Fetal /embryonal</td>
<td>dc+dn</td>
<td>cyto</td>
<td>32D&gt;Y</td>
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<td>Pure fetal</td>
<td>dc</td>
<td>cyto</td>
<td>37S&gt;Y</td>
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<td>dc</td>
<td>cyto</td>
<td>34G&gt;V</td>
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<td>dn</td>
<td>nuc</td>
<td>32D&gt;N</td>
</tr>
<tr>
<td>CCRG64(^a)</td>
<td>Epithelial</td>
<td>Fetal /embryonal</td>
<td>dc+fn</td>
<td>neg</td>
<td>32D&gt;V</td>
</tr>
<tr>
<td>CCRG64(^b)</td>
<td>Epithelial</td>
<td>Pure fetal</td>
<td>fn</td>
<td>neg</td>
<td>32D&gt;V</td>
</tr>
<tr>
<td>CCRG65</td>
<td>Epithelial</td>
<td>Pure fetal</td>
<td>dn</td>
<td>neg</td>
<td>34G&gt;R</td>
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<td>dc</td>
<td>cyto</td>
<td>34G&gt;E</td>
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<td>dc+fn</td>
<td>cyto</td>
<td>32D&gt;V</td>
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<td>dc+fn</td>
<td>cyto</td>
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<td>fc+fn</td>
<td>cyto</td>
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<td>dc+dn</td>
<td>cyto</td>
<td>35I&gt;S</td>
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<td>dc+dn</td>
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<td>32D&gt;V</td>
</tr>
</tbody>
</table>

**Abbreviations:** IHC, immunohistochemistry, dc, diffuse cytoplasmic, dn, diffuse nuclear, fc, focal cytoplasmic, fn, focal nuclear, cyto, cytoplasmic.
**Figure 4.5:** IHC using an antibody to detect tyrosine-phosphorylated β-catenin. A shows negative staining with Y654-β-catenin antibody on a sample of HB tumour. A haematoxylin counterstain was used to stain the nuclei blue. B shows diffuse cytoplasmic staining of Y654-β-catenin in light brown against blue nuclei and C shows nuclear and cytoplasmic staining of hepatoblastoma with an antibody to Y654-β-catenin.
4.2.5 Analysis of β-catenin and Cyclin D1 reveals a correlation between Y654-β-catenin and Cyclin D1

We also examined the correlations between expression of total β-catenin, Y654-β-catenin and CTNNB1 mutations with Cyclin D1 expression. Cyclin D1 is a transcriptional target of β-catenin involved in cell cycle and implicated in cancer. We used Fisher’s Exact test to analyse the data. No significant correlation was found between total β-catenin and Cyclin D1 or CTNNB1 and Cyclin D1 (\(P=0.16, P=0.45\) respectively). However, analysis of Y654-β-catenin revealed a statistically significant correlation between nuclear localization of the protein and Cyclin D1 expression (\(P=0.03\)).

<table>
<thead>
<tr>
<th></th>
<th>Nuclear/cytoplasmic</th>
<th>Cytoplasmic only</th>
<th>Nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total β-catenin</td>
<td>18/20 (90%)</td>
<td>14/20 (70%)</td>
<td>1/20 (5%)</td>
</tr>
<tr>
<td>Negative</td>
<td>5/20 (25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Possible deletion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation</td>
<td>67/78 (85%)</td>
<td>44/78 (57%)</td>
<td>27/78 (34%)</td>
</tr>
<tr>
<td>Wild Type</td>
<td>7/78 (9%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.6: Frequency of total β-catenin and Y654-β-catenin expression in our Hepatoblastoma cohort. Mutated and samples and those with possible deletions in the CTNNB1 gene were grouped together for the purposes of this analysis and protein expression compared to that of wild type HB samples.
4.2.6 Cell line expression of total $\beta$-catenin and Y654-$\beta$-catenin in response to HGF activation mirrors that of HB tumours

4.2.6.1 HuH-6 and HuH-7 cell lines
To validate the IHC results from the hepatoblastoma tumour samples we sought to replicate the findings in cell lines. The cell lines chosen were HuH-6 and HuH-7. HuH-6 is a hepatoblastoma cell line originally derived by Doi et al in 1976 (Doi, 1976). The cell line secretes plasma proteins such as albumin, fibronectin and fibrinogen, commonly produced in the liver, and also expresses $\alpha$-fetoprotein (Bagnarelli and Clementi, 1987). HuH-6 cells are adherent epithelial cells routinely used in research. A mutation in the $CTNNB1$ gene, coding for $\beta$-catenin has previously been reported in the HuH-6 cell line. We also used the HuH-7 cell line in our experiments. This hepatoma cell line is derived from a Japanese adult with well-differentiated HCC (Nakabayashi et al., 1982). It also forms adherent layers in culture and secretes serum proteins and $\alpha$-fetoprotein like HuH-6. Neither cell line has tested positive for viral antigens.

4.2.6.2 $CTNNB1$ status of HuH-6 and HuH-7
We sought to confirm the reported $CTNNB1$ status of our two cell lines by DNA sequence analysis. Human hepatoblastoma cells, HuH-6 (JCRB) were routinely maintained in minimum essential media (MEM) containing 10% FBS and penicillin/streptomycin. The human hepatoma cell line HuH-7 (JCRB) was cultured in Dulbecco’s Minimum Essential Media (D-MEM) with 10% FBS and penicillin/streptomycin. After routine culture and passage of the cells, RNA was extracted from both cell lines. The RNA was analysed for $CTNNB1$ mutations in exon 3 using RT-PCR and sequencing as outlined in Materials and Methods. We found that the hepatoblastoma cell line, HuH-6, carries a missense mutation of G34>V, a known variant of $\beta$-catenin while sequencing of the hepatoma cell line, HuH-7 revealed wild type $CTNNB1$ (Figure 4.11). These results were consistent with expectations and provided us with a control cell line in our investigations.
Figure 4.7: Direct sequence analysis of exon 3 of β-catenin in HuH-7 and HuH-6 cell lines. HuH-6 carries a G→T transversion, resulting in a glycine to valine amino acid change in codon 34. HuH-7 displays wild type β-catenin.

4.2.6.3 Treatment of Cell lines with Hepatocyte Growth Factor (HGF)

We sought to activate β-catenin in our cell lines by treatment with HGF for different lengths of time. Binding of HGF to its receptor, c-Met, is known to phosphorylate β-catenin at specific tyrosine residues and lead to its accumulation and activation. The HuH-6 and HuH-7 cells were serum starved for 24 hours prior to treatment with recombinant human HGF to a concentration of 50ng/ml for 30, 60, 90 and 120 minutes. Nuclear and cytoplasmic protein fractions were isolated from the cell lines at the various timepoints indicated.
Figure 4.8: BCA assay for nuclear proteins. Protein concentrations and spectrophotometric values of BSA standards used to generate a standard curve. Protein concentrations from nuclear extracts of cell lysates are calculated from the standard curve.

The nuclear and cytoplasmic protein concentrations were determined by bicinchoninic acid protein assay using BSA as a standard on a SpectraMax 190 spectrophotometer using SoftMax version 5.9 software (Figures 4.8 and 4.9 respectively). This allowed us to load equal amounts of protein (20µg) into each well of subsequent protein separation gels.
Figure 4.9: BCA assay for cytoplasmic proteins. Protein concentrations and spectrophotometric values of BSA standards used to generate a standard curve. Protein concentrations from cytoplasmic extracts of cell lysates are calculated from the standard curve.

4.2.6.4 Immunoblot analysis of proteins after timecourse experiment

The separated proteins were transferred onto a nitrocellulose membrane by western blotting. The immunoblots were then probed using antibodies to total β-catenin and Y654-β-catenin. Figure 4.10 shows the immunoblot of the timecourse experiment using the total β-catenin antibody. Immunoblot analysis of the nuclear and cytoplasmic fractions showed β-catenin expression in untreated HuH-6 (T0) cells, which may be due to the activating mutation in exon 3 of the CTNNB1 gene. The level of total β-catenin increased slightly through each timepoint peaking at 90 minutes. Total β-catenin was almost undetectable in the nuclei of untreated HuH-7 cells which is wild type for CTNNB1, and the level seen in the cytoplasm is
noticeably lower than that of HuH-6 cells. Immunoblotting with anti-TBP for nuclear proteins and anti-β-actin for cytoplasmic extract was used to confirm equal loading.

**Figure 4.10: Western blot to detect total β-catenin levels after timecourse experiment.** β-catenin expression in the cytoplasm and nucleus of HuH-6 and HuH-7 cells after timecourse experiment with HGF treatment at various timepoints. Time 0 (T0) is the negative control.

We probed the blots using an antibody to Y654-β-catenin which allowed us to determine how much of the nuclear β-catenin was due to activation by HGF rather than an activating CTNNB1 mutation. No tyrosine-phosphorylated β-catenin was seen in untreated cell fractions, while very low levels of the phosphorylated protein were detected in HuH-6 nuclei and cytoplasm compared with HuH-7 after HGF treatment (Figure 4.11). These results show distinct mechanisms of β-catenin translocation and nuclear accumulation in the cell lines with and without CTNNB1 mutations and serve to corroborate our immunohistochemical findings.
Figure 4.11: Western blot to examine $\beta$-catenin and Y654-$\beta$-catenin expression
Immunoblots of nuclear and cytoplasmic protein fractions from HuH-6 and HuH-7 cells probed with total $\beta$-catenin and Y654-$\beta$-catenin before and after treatment with HGF for 90 minutes. Blots were stripped and reprobed with $\beta$-actin and TBP to ensure equal loading of protein lysates.

4.3 Discussion
The accumulation of $\beta$-catenin appears to be a crucial event in the tumorigenesis of HB and although $\beta$-catenin gene mutations have been widely reported in HB, the levels to which they are seen and to which they correlate with protein accumulation vary widely (Table 4.2). Mutation frequencies of up to 70% have been reported, with deletions in exon 3 accounting for up to half of these (Buendia, 2002, Koch et al., 1999, Takayasu et al., 2001, Udatsu et al., 2001). These studies used deletion screening by PCR, and direct sequencing on a variety of fresh tissue and formal-fixed tumours. However we, and other groups, have shown a lower frequency of mutations/deletions (21%) but a high level of $\beta$-catenin accumulation (87%) in our sample group (Curia et al., 2008, Inukai et al., 2004, Park et al., 2001b). Our results serve to underline the importance of $\beta$-catenin activation in the pathogenesis of the tumour but the discrepancy in mutation frequencies implies that an alternative activation of $\beta$-catenin may occur. This may be explained by mutations in other Wnt
pathway members (Koch 2005). Lopez-Terrada et al identified differential wnt and notch pathway activation in different histologic subtypes (Lopez-Terrada et al., 2009).

Table 4.2. Review of published β-catenin mutation/expression studies including our own work highlighted.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Mutation frequency</th>
<th>Deletion frequency</th>
<th>Protein accumulation</th>
<th>References</th>
</tr>
</thead>
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<td>34%</td>
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Danilkovitch-Miagkova et al demonstrated that c-Met tyrosine phosphorylation of β-catenin has the same downstream effect on transcription as activation of β-catenin through the Wnt pathway, and further studies have implicated c-Met activation of β-catenin in cancer pathogenesis (Maulik et al., 2002b, Breuhahn et al., 2006). More recently, Cieply et al investigated hepatocellular (HCC) tumour characteristics occurring in the presence or absence of mutations in CTNNB1. The authors found that the fibrolamellar (FL) tumours had the highest tyrosine-654-phosphorylated-β-catenin (Y654-β-catenin) levels in the study and these tumours also lacked mutations in the CTNNB1 gene (Cieply et al., 2009).

This prompted us to analyse our samples for c-Met related β-catenin protein activation. We used an antibody to detect tyrosine-654 phosphorylated β-catenin (Y654-β-catenin) as a surrogate marker for HGF/c-Met activation. Using this method we found that a large proportion of our cohort showed c-Met related β-catenin protein activation. Statistical analysis of tumour groups with and without mutations shows a
significant correlation between wild type β-catenin and nuclear accumulation of Y654-β-catenin. This is in keeping with the findings of Cieply et al in hepatocellular carcinoma. To validate our tumour findings, we looked at the effects of HGF treatment on β-catenin and Y654-β-catenin in two liver cancer cell lines, with and without CTNNB1 mutations. The results reflected those seen in HB tumours with c-Met activated β-catenin found only in the cell line with wild type CTNNB1 following HGF treatment. It must be noted, however, that nuclear Y654 β-catenin was seen in two tumours carrying mutations/deletions so an overlap of activation pathways may occur. Furthermore thirteen tumours harbouring mutations/deletions also showed Y654 β-catenin expression in the cytoplasm. Further studies must be carried out to ascertain the effect of mutated β-catenin on the nuclear accumulation of the c-Met related β-catenin pool.

Figure 4.12. HB samples with aberrant β-catenin expression showing the breakdown of samples with gene mutations/deletions and Y654-β-catenin protein expression.
Overall analysis of tumours with aberrant β-catenin expression revealed only a small percentage (5%) that has neither mutations in the CTNNB1 gene nor expression of tyrosine654-phosphorylated β-catenin (Figure 4.12). These tumours may have mutations in other genes such as AXIN or APC that lead to abnormal β-catenin accumulation or activation through a different pathway. These findings underline that aberrant activation of β-catenin may be critical to the pathogenesis of HB but the means of this activation may not be as important as was previously thought.

Interestingly our results also show a novel correlation between nuclear accumulation of Y654-β-catenin and the cell-cycle regulator, Cyclin D1. Previous studies in HB have reported correlations between β-catenin and its transcriptional target, Cyclin D1 (Takayasu et al., 2001). However, our data shows that Cyclin D1 expression does not correlate with total β-catenin expression in our HB cohort but is significantly correlated with Y654-β-catenin. This may indicate that downstream effects of β-catenin are dependant on its mode of activation, contrary to the current model where an increase in β-catenin signalling is thought to cause an increase in Cyclin D1 activity regardless of the upstream signalling. Although its mode of activation in tumorigenesis is not well understood it may be regulated by a feedback-loop with miR-17/20 (Yu et al., 2008). Its overexpression has been tightly linked to pathogenesis and also has a prognostic role in many cancers including breast cancer (Arnold and Papanikolaou, 2005). Takayasu et al found that overexpression of Cyclin D1 correlated to aberrant β-catenin activation and was associated with poorly differentiated tumours in their study of hepatoblastomas {Takayasu, 2001 #43}. A common polymorphism found in CCND1, the gene coding for Cyclin D1, is associated with increased risk and poor prognosis in certain cancers (Arnold and Papanikolaou, 2005, Howe and Lynas, 2001). This polymorphism was investigated in hepatoblastoma and found to correlate with a younger age of onset of the disease (Pakakasama et al., 2004). Taken together with our findings this indicates a possible role for Cyclin D1 in HB pathogenesis that warrants further investigation.

Although treatment of HB with cisplatin or PLADO followed by resection is highly successful there remains >15% of HB that suffer from relapse. These relapse patients are often refractory to conventional chemotherapy and have a survival rate of <20%.
This is due to the acquired drug resistance seen in most HB tumours after four or five cycles of chemotherapy (von Schweinitz et al., 1997a). Increased MDR1 gene expression levels and Bcl-2 mediated chemoresistance have been investigated as possible causes of treatment failure (Warmann et al., 2002, Warmann et al., 2008). A recent study by Grotegut et al reported HGF/c-Met activation of PI3K/AKT resulting in chemotherapeutic resistance in HB cells, and taken together with our results highlights the importance of HGF/c-Met as a candidate target for therapy in HB.

The translation of our findings may be important for design of future clinical trials, identifying patients for individual targeted therapy and allowing for fewer side effects. Additionally, β-catenin itself may be a promising target for therapy. Inhibition of β-catenin in HuH-6 and HepG2 cell lines by RNAi leads to a decrease in c-Myc and Cyclin D1 and a transient decrease in proliferation (Sangkhathat et al., 2006). However, no change in target genes or proliferation was seen in HuH-7 cells under the same conditions, implying that the mutation status of CTNNB1 may be important in the RNAi pathway.

Our findings may also have a wider application in the treatment of other tumour types that display β-catenin activation without associated gene mutation. Somatic mutations in exon 3 of the β-catenin gene have been reported in a variety of cancers (Morin, 1999, Polakis, 2000). However, aberrant accumulation of the β-catenin protein without activating mutations have been reported in a subset of many cancers such as gastrointestinal carcinoid tumour, ovarian cancer, cutaneous lymphoma, malignant melanoma and pancreatic adenocarcinoma (Bellei et al., 2004, Fujimori et al., 2001, Rimm et al., 1999, Wright et al., 1999, Zeng et al., 2006b). HGF/c-Met activation of β-catenin may account for the discrepancies between gene mutation and protein expression seen in these tumours and this could indicate susceptibility to RTK inhibitors in the treatment regimen.
Chapter Five
Analysis of miRNA expression in
Hepatoblastoma
Chapter 5: Analysis of microRNA expression in hepatoblastoma

5.1 Introduction

The field of miRNA research has rapidly expanded in the past fifteen years since the first reporting of this novel mechanism of epigenetic regulation (Lee et al., 1993). Now miRNAs are believed to be involved at all levels of cellular regulation. The contribution of miRNAs has taken centre stage in cancer research since they were shown to be functional in humans (Bartel, 2004). Although research is still at the early stages, major findings that have contributed to the understanding of the role of miRNAs in cancer progression have been made in the past eight years including the regulation of tumour suppressor genes and oncogenes by miRNAs. For example, the anti-apoptotic oncogene Bcl-2 is directly targeted and inhibited by miR-15a, while miR-21 may positively regulate Bcl-2 and promote tumourigenesis (Cimmino et al., 2005, Si et al., 2007). Only a very small fraction of the putative thousands of miRNAs have been annotated to date, but miRNA array profiles have proven to be more accurate than gene expression arrays for diagnosis in different tumours (Lu et al., 2005). Many recent studies have found that these small non-coding RNAs can predict tumour behaviour in a variety of malignancies including gastric cancer, pancreatic cancer and neuroblastoma (Chen and Stallings, 2007, Li et al., Garzon et al., 2009) showing that miRNAs may also have prognostic use in cancer. In addition to diagnosis and prognostic use, miRNAs present exciting new opportunities for targeted cancer therapy.

Given the role of miRNAs in normal developmental pathways and that paediatric tumours tend to involve misregulation of these pathways, it seems very likely that miRNAs play an important role in paediatric cancer pathogenesis. Array profiling of miRNAs in medulloblastoma has identified signatures that correlate with molecular subgroups and response to treatment (Fernandez et al., 2009). Extensive research into the role of miRNAs in neuroblastoma has identified miR-34a as a potent tumour suppressor by targeting MYCN (Cole et al., 2008). With the exception of these two tumours, the role of miRNA in paediatric solid tumour development and regulation has not been widely studied to date.
Although the role of miRNAs in the pathogenesis of HB has not been widely investigated, the contribution of miRNAs to normal liver development and HCC has been firmly established. The first miRNA to be identified in the liver was miR-122 (Moroy et al., 1989) and it is expressed at every stage of liver development and also in hepatic cell lines. Two separate studies have linked miR-122 to cholesterol biosynthesis (Krutzfeldt et al., 2005, Esau et al., 2006). While miR-122 appears as the most highly expressed miRNA in adult liver, miR-92a and miR-483 seem to be specifically expressed in fetal liver (Fu et al., 2005). Table 5.1 details miRNAs expressed in fetal and mature liver from a review of the literature (Girard et al., 2008).

Differential miRNA expression patterns seen between fetal and adult liver imply differing roles for miRNAs during normal liver development. For example miR-451 and miR-92 were shown to be abundantly expressed in fetal versus adult liver and these miRNAs have roles in erythropoiesis and cell cycle-related gene regulation respectively (Tzur et al., 2009). Conversely, this study found that miRNAs such the let-7 family members and miR-23b are expressed predominantly in adult livers and these are capable of inhibiting cell proliferation (Tong et al., 2009) and may lead to cell cycle arrest in mature hepatocytes.

Many HCC miRNA array studies have been carried out worldwide since the technology became available but show very limited overlap in results. This may be due to virus-mediated disease and different geographical locations and ethnic groups of the studies, as this has a huge impact on HCC development. A meta-analysis of miRNA studies in HCC found that particular miRNAs were overexpressed including let-7a, miR-21, miR-221, miR-222, miR-224 and miR-301 in more than one study and miR-122a, miR-125a, miR-139, miR-145, miR-199a, miR-200b and miR-223 were significantly down-regulated in many HCCs studied (Varnholt, 2008). Many of these miRNAs have validated functions in regulating TSGs and oncogenes, such as suppression of the tumour suppressor gene, PTEN by miR-21 (Meng et al., 2007a).
Table 5.1: Repertoire of miRNAs in human liver (Girard et al., 2008).

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</table>

The left column corresponds to the most up-to-date compilation in the liver referred to as "atlas liver" (Landgraf, 2007 #1473), the middle column to "adult liver" (Barad, 2004 #1474), and the right column to "fetal liver" (Fu et al., 2005). "++" and "++" signs indicate the levels of expression of the various miRNAs that were assessed from the different studies. The minus sign is used to indicate very low to undetectable levels, one plus to three pluses indicate a gradual expression from low levels to very high levels. The slash stands for the miRNAs that were not assayed.
Restoring the expression of miRNAs suppressed in tumours represents a promise for effective anti-cancer treatment. Therapeutic use of miRNAs by RNA interference (RNAi) is currently being investigated by several groups. It has the potential advantage of needing low concentrations, limited toxicity and high target specificity (Kerr and Davidson). Initial studies reported hepatotoxicity and off-target effects in animal models (Kerr and Davidson) but a recent study by Kota et al reported for the first time that replacement of a single microRNA, miR-26a, had relevant anti-tumour effects in a mouse model (Kota et al., 2009). MiR-26a, normally expressed in liver cells is lost in HCC and its reintroduction lead to inhibition of cancer cell proliferation and induced tumour cell apoptosis in mice liver in this study. Specific targeting of endogenous miRNAs also holds therapeutic promise. Targeting of miR-122 with specific anti-miR oligonucleotides has lead to specific, dose-dependant miR-122 silencing without hepatotoxicity in mice (Krutzfeldt et al., 2005). This type of specific miRNA targeting could be enormously useful in the inhibition of miRNAs such as miR-214, known to contribute to drug resistance in cancer (Yang et al., 2008).

5.2 Results

Our study spans over four years of advances in the field of miRNA research. We first used a probe set from Ambion containing a limited numbers of probes available at the time. We validated these arrays with miRNA extracted from mouse liver before use with human tumour samples. For this study we accessed FFPE tumour samples. RNA from FFPE tissue is usually very fragmented and this makes it suitable for small RNA analysis. To analyse miRNAs from FFPE tissue we found that it was necessary to use a system of miRNA amplification prior to Ambion array analysis, as this system required a large concentration of starting material. Our most recent efforts used Agilent arrays, which contain almost five-times the number of human miRNA probes than the Ambion arrays used initially and need only tiny quantities of starting RNA with no amplification required.

5.2.1 Validation of Ambion probe set with mouse liver miRNAs

Our initial miRNA probe set was one of the first miRNA array probe sets available commercially. This mirVana™ miRNA probe set (Ambion) consists of 768 spots containing a panel of 384 unique miRNA probes from rat, mouse and human found in
the miRNA registry at the time of manufacture, October 2005:
microrna.sanger.ac.uk/sequences/index.shtml

To test whether miRNA from formalin-fixed tissue would be appropriate for array analysis we validated the arrays with miRNA extracted from both fresh mouse liver and formalin-fixed, paraffin-embedded mouse liver from matched specimens. RNA concentrations extracted from the samples are detailed in Table 5.2. The concentration of the RNA in the total sample volume (30µl) was also calculated. We used Ambion's flashPAGE™ Fractionator System for the isolation of mature miRNAs from total RNA. The flashPAGE™ System protocol recommends using 1 to 100 µg RNA so we loaded total volumes of our samples for fractionation. After isolation of the small RNA fraction and post-fractionation clean-up, the RNA concentration was again measured and this is also detailed in Table 5.2. A minimum of 500ng of miRNA was needed for each labelling reaction and as our concentrations were very low we decided to pool the three FFPE samples and the three fresh samples together.

Table 5.2 Mouse liver samples used in miRNA array analysis showing RNA and miRNA concentrations.

<table>
<thead>
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<th>RNA ng/µl</th>
<th>RNA µg/30µl</th>
<th>miRNA ng/µl</th>
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<td>100</td>
<td>3000</td>
</tr>
<tr>
<td>ML2-F</td>
<td>330</td>
<td>9.9</td>
<td>37.7</td>
<td>1131</td>
</tr>
<tr>
<td>ML3-F</td>
<td>250</td>
<td>7.5</td>
<td>3.5</td>
<td>105</td>
</tr>
</tbody>
</table>

ML = mouse liver, PE = paraffin-embedded tissue, F= fresh tissue

The miRNA samples were labelled using the mirVana™ miRNA labelling kit (Ambion) using Cy3 dye to label pooled sample from fresh mouse liver and Cy5 to label the FFPE miRNA. The samples were hybridized to the array and the array slide scanned as detailed in Chapter 2, Section 17.3.
5.2.2 Results and recommendations from mouse liver validation array
The signal intensity was low from most probes as shown in Figure 5.1 but the relative levels of the miRNAs from the two samples appear to be very similar. No miRNAs appear to be represented more strongly in either sample with any significance. The Positive Control miRNAs are the only array spots to appear visually different showing that hybridization has worked well. These results indicated that miRNA extracted from FFPE tissue generated array results that are well correlated with those from fresh tissue. However, the concentrations of total RNA from hepatoblastoma samples were insufficient for flashPAGE™ fractionation and subsequent labelling with the mirVana™ miRNA labelling kit.

Figure 5.1: Subsection of Ambion microRNA array. Positive control spots appear orange/red.

5.2.3 Amplification of miRNA from total hepatoblastoma RNA
Fortunately, during the course of this work, a system for amplification of miRNAs was developed. The NCode™ miRNA Amplification System (Invitrogen) amplifies senseRNA molecules from minute quantities of purified miRNA. The system generates >1000-fold amplification of miRNAs and preserves the relative abundance of the different miRNAs, which allows quantitative comparison in downstream
experiments such as array analysis. This system uses up to 30ng of miRNA isolated from as little as 300ng of total RNA. We used the PureLink™ miRNA isolation kit (Invitrogen) to isolate miRNA from our original HB RNA samples and amplified the miRNA with the NCode™ miRNA Amplification System. We then proceeded with labelling and probe hybridization as detailed above.

5.2.4 Array analysis of miRNA from hepatoblastoma tissue

In this analysis nine spotted microarrays were used, with pooled microRNA from normal adult, normal fetal and tumour tissue as reference (Cy3) and the test samples consisting of miRNA from five HB tumours and four adjacent normal tissue samples (Cy5). The sample set contained six biological replicates consisting of three matched pairs (tumour and normal samples from HB5, HB7, HB10) in order to make a comparison of tumour versus normal tissue. In addition to these matched pairs there were two further tumour samples, HB3 and HB4 and one sample of adjacent normal tissue from HB2.

5.2.4.1 Visualization of array data

The density plots showed good separation of background and foreground intensity in the red channel (test) of each array. For the green (reference) channel, this was not the case, however, with most arrays showing a high level of green background intensity (Figure 5.2).

Figure 5.2: Density plots for sample HB5T. The log² intensity distributions of the foreground and background are plotted for each of the red and green channels for HB5T (foreground is bright, background is pale).
The boxplots and scatterplots showed that background correction has little effect on the distribution of the log fold-changes (the M-values) for each array, although, if anything, the distributions became slightly more variable (Figure 5.3). This is most likely due to the high green background observed on each array.

The MA plots illustrated that background correction produces much more of a random scatter than is observed in the MA plots without background correction (Figure 5.4 A+B). A strong linear relationship between A and M was also observed.
when no background correction is applied. Global relationships between A and M are generally the result of a dye effect, which is removed by normalization. In our array study the relationship is such that low intensity spots tend to be green (negative M values), while high intensity spots tend to be red (positive M values). The effect seen here is much stronger than would usually be encountered with spotted mRNA arrays, and may indicate problems with either the labelling or hybridization. Based on all of these observations, it was decided that background correction was unlikely to provide an advantage in this analysis.

Figure 5.4: Scatterplots for HB10N and HB10T. A shows MA plots pre-normalization with no background correction for samples HB10N and HB10T. B shows the data with background correction. C shows the data post-normalization for the two samples.

5.2.4.2 Array Normalization

The limma package was used within the R computing environment for data normalization. This was carried out by Dr. Mik Black from the Biochemistry Department, University of Otago, Dunedin. Based on the observations made from
analysing the visual data, background correction was not performed. Within each array, global loess normalization was used. This is a single local regression line fit to the MA plot of each array. Between arrays, scale normalization was used to obtain similar M-value densities across arrays.

Figure 5.5: Boxplots after normalization A. Boxplot of data for all arrays after within-array normalization and B. after within/between array normalization.

The within- and between-array normalization methods are designed to produce MA plots that are centred and roughly symmetric about zero on the y-axis (the M values,
or log fold-changes), and are similar across arrays. The theory behind this is that most probes on the array are not expected to exhibit differential expression (i.e. log fold-change of 0), and those that do are equally likely to be either up- or down-regulated. The MA plots seen here are generally noisier than standard microarray experiments, although this may reflect the relatively small number of probes (384) on each array. Arrays HB2 (normal) and HB10 (tumour) do not exhibit good spread along the A (average expression) axis, which may be indicative of poor quality (Figure 5.4).

5.2.4.3 Analysis of miRNA array data

The limma package was used to fit a linear model to the data in order to detect probes undergoing significant differential expression between normal and tumour tissues. The model accounts for duplicate spots, but not for the repeated measures made on samples HB5, HB7 and HB10. The likely consequence of this is that the results produced are slightly over-optimistic, since treating these correlated samples (which come from the same patient) as coming from different patients implies the presence of more biological variability really exists. Table 5.2 shows the ten genes most highly ranked by the linear model.

Table 5.3: The ten highest ranking miRNAs identified by array analysis

<table>
<thead>
<tr>
<th>ID</th>
<th>M</th>
<th>FC</th>
<th>A</th>
<th>t.stat</th>
<th>P.Value</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>rno_miR_343</td>
<td>-0.481</td>
<td>0.717</td>
<td>9.599</td>
<td>-3.102</td>
<td>0.005</td>
<td>0.989</td>
</tr>
<tr>
<td>rno_miR_333</td>
<td>-0.406</td>
<td>0.755</td>
<td>9.220</td>
<td>-2.907</td>
<td>0.008</td>
<td>0.989</td>
</tr>
<tr>
<td>rno_miR_151_AS</td>
<td>-0.357</td>
<td>0.781</td>
<td>9.175</td>
<td>-2.443</td>
<td>0.023</td>
<td>0.989</td>
</tr>
<tr>
<td>ambi_miR_7092</td>
<td>-0.425</td>
<td>0.745</td>
<td>8.981</td>
<td>-2.378</td>
<td>0.026</td>
<td>0.989</td>
</tr>
<tr>
<td>hsa_miR_190</td>
<td>-0.385</td>
<td>0.766</td>
<td>8.833</td>
<td>-2.359</td>
<td>0.027</td>
<td>0.989</td>
</tr>
<tr>
<td>ambi_miR_7090</td>
<td>-0.385</td>
<td>0.766</td>
<td>9.124</td>
<td>-2.094</td>
<td>0.048</td>
<td>0.989</td>
</tr>
<tr>
<td>ambi_miR_7082</td>
<td>-0.451</td>
<td>0.732</td>
<td>9.206</td>
<td>-2.080</td>
<td>0.049</td>
<td>0.989</td>
</tr>
<tr>
<td>hsa_miR_370</td>
<td>-0.314</td>
<td>0.805</td>
<td>9.091</td>
<td>-1.886</td>
<td>0.072</td>
<td>0.989</td>
</tr>
<tr>
<td>hsa_miR_15a</td>
<td>0.272</td>
<td>1.208</td>
<td>9.265</td>
<td>1.883</td>
<td>0.073</td>
<td>0.989</td>
</tr>
<tr>
<td>ambi_miR_7075</td>
<td>0.370</td>
<td>1.293</td>
<td>9.302</td>
<td>1.869</td>
<td>0.075</td>
<td>0.989</td>
</tr>
</tbody>
</table>

ID is the name of the probe, M is the log2 fold-change (negative indicates down-regulation), FC is the fold-change, A is the average intensity, t.stat is the limma-moderated t-statistic, P.Value is the unadjusted p-value for each probe, and adj.P.Val is the p-value adjusted for multiple comparisons (based on the number of probes being investigated).
Based on the results presented above, no miRNA probes pass the criteria for statistical significance (adjusted $p$-value less than 0.05), which guarantees a false discovery rate (FDR) of less than 5%.

**Figure 5.6: Volcano plot of log fold-change versus likelihood of differential expression (log odds).**

However, an inspection of the volcano plot, which plots the log fold-change *versus* likelihood of differential expression, reveals that the top seven probes appear to sit somewhat separate from the others, and thus suggests that these *may* be differentially expressed between normal and tumour tissues. As all seven M-values were negative they appear to be down-regulated in the tumour. Figure 5.7 shows a plot of the top seven probes showing the $\log^2$ expression intensity for the duplicate probes (connected by pale solid lines) in the tumour (red) and normal (blue) samples. Tumour and normal samples from the same patient are connected with solid gray lines. Means values for the tumour and normal groups are indicated by bright red and blue dots respectively. The fold-change for each probe is shown beneath the probe name. Here, negative values indicate down-regulation of tumour relative to normal, so a value of -2 indicates that expression is twice as high, on average, in the normal
tissue for that probe. This is equivalent to a fold-change of 0.5 in the output table.

Figure 5.7: Normalized data showing the top seven probes plotted against the log fold-change.

The equivalent plot for the unnormalized data clearly shows the effect that normalization has had on the data (Figure 5.8). All of the average differences are now much smaller i.e. the means are closer together, and most of the relationships have reversed with tumour expression higher than normal tissue expression. This is most likely due to the strong linear relationship between M and A on each array that was noted above. Given that the results are heavily dependent on the normalization method that was used, and the fact that statistical significance was not achieved, there is only rather weak evidence for differential expression of these probes.
Figure 5.8: Unnormalized data showing the top seven probes plotted against the log fold-change.

5.2.4.4 Evaluation of miRNA array data from Ambion probe set
Despite the limited evidence for differential miRNA expression, we carried out a closer inspection of the top seven miRNAs differentially expressed between tumour and normal tissue in hepatoblastoma. This revealed that three of the miRNAs correspond to probes from the rat genome (*Rattus norvegicus*) and were discounted from further analysis. Of the remaining four, only one had a designated human miRNA name, hsa-miR-190 and the other three at the time of array printing (2006) were unique putative miRNA probes designated ambi-miR-7092, 7090 and 7082 by the probe manufacturers. In the intervening time these probes, 7092, 7090 and 7082 have been redesignated as hsa-miR-503, hsa-miR-511 and hsa-miR-202* respectively. Of greatest interest to our study is miR-503. This microRNA has been implicated in pancreatic development and muscle differentiation (Joglekar et al., 2007, Sarkar et al.). More relevant to cancer research is the involvement of miR-503 in retinoblastoma pathogenesis and the development of parathyroid and adrenocortical tumours (Corbetta et al., Tombol et al., 2009). A study by Jiang et al has proposed that miR-503 works as a tumour suppressor interacting with Cyclin D1 and causing cell growth inhibition (Jiang et al., 2009). Experimental validation showed that miR-
503 could suppress CCND1 at both the mRNA and protein level. Our data does not identify any miRNAs in common with those found in the HB study by Magrelli et al (Magrelli et al., 2009). This study analysed three matched pairs of HB tumour and normal tissue by miRNA array and validated several of the miRNAs in a further six matched samples. The small number of samples in both of these studies makes it very difficult to draw any meaningful conclusions from the data.

5.2.5 miRNA array analysis using the Agilent array system

As miRNAs are such a recent discovery the technology used to study them has evolved very quickly over the last five years. Early miRNA array probe sets are almost obsolete and current array slides contain probes to more than 800 human miRNAs. The technology used today is also less cumbersome, has increased sensitivity and uses tiny quantities of starting material. For our further array experiments we used the Human miRNA Microarray Version 3 (Agilent) that contains probes for 866 human and 89 human viral microRNAs from the Sanger database v12.0. Agilent's unique labelling method uses 100ng of total RNA extracted from HB samples using mirVana™RNA extraction kit (Ambion) without the need for miRNA isolation or amplification. This method has been shown to span a linear range from 0.2 amol to 2 fmol of input miRNA and is suitable for use with FFPE samples (Wang et al., 2007).

5.2.5.1 HB samples for miRNA array analysis

The Agilent microarray slides we used were of 8 x 15K format. This means that eight samples were run on a single slide minimising inter-slide variability. For the initial analysis we chose to look for variation in miRNA expression patterns between HB samples with wild type CTNNB1 and those with mutations, following on from our results in the previous two chapters. We chose five samples with CTNNB1 mutations, two WT samples and one sample of normal fetal liver (non-hepatoblastoma). Clinical and IHC data for the seven HB samples is given in Table 5.3.
Table 5.4: Clinical and molecular characteristics of HB patients used in the initial Agilent miRNA microarray experiment.

<table>
<thead>
<tr>
<th>CCRG</th>
<th>Type</th>
<th>Subtype</th>
<th>Pretext</th>
<th>EFS</th>
<th>CTNNB1</th>
<th>CyclinD1</th>
<th>Y654</th>
</tr>
</thead>
<tbody>
<tr>
<td>16T</td>
<td>epith/mesench</td>
<td>Mixed</td>
<td>2</td>
<td>1</td>
<td>32D&gt;Y</td>
<td>pos</td>
<td>cyto</td>
</tr>
<tr>
<td>48T</td>
<td>epithelial</td>
<td>Fetal</td>
<td>3</td>
<td>0</td>
<td>37S&gt;Y</td>
<td>neg</td>
<td>cyto</td>
</tr>
<tr>
<td>51T</td>
<td>epith/mesench</td>
<td>Fetal</td>
<td>3</td>
<td>0</td>
<td>Wild type</td>
<td>neg</td>
<td>cyto + nuc</td>
</tr>
<tr>
<td>55aT</td>
<td>unknown</td>
<td>unknown</td>
<td>2</td>
<td>1</td>
<td>Wild type</td>
<td>pos</td>
<td>cyto + nuc</td>
</tr>
<tr>
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<td>Fetal</td>
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<td>0</td>
<td>32D&gt;V</td>
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<td>neg</td>
</tr>
<tr>
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<td>epith/mesench</td>
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<td>0</td>
<td>34G&gt;E</td>
<td>neg</td>
<td>cyto</td>
</tr>
<tr>
<td>88T</td>
<td>epith/mesench</td>
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<td>3</td>
<td>1</td>
<td>32D&gt;V</td>
<td>neg</td>
<td>cyto</td>
</tr>
</tbody>
</table>

5.2.5.2 Controls for Agilent arrays

Agilent's microRNA Spike-In kit consists of two microRNA Spike-In solutions for process control. The Spike-In solutions are used to help distinguish significant biological data from processing issues. The Labelling Spike-In solution was spiked into the labelling reaction while the Hyb Spike-In solution was spiked into the hybridization reaction. As these are single colour arrays, the samples are compared to each other during the analysis and not to a control sample as was carried out in previous array experiments.

5.2.5.3 Feature Extraction and Quality Control of array data

Array slides were scanned using the Agilent Microarray Scanner version C with ScanControl software version 8.X and default settings for miRNA analysis. Unlike our previous microarray experiments where feature analysis and grid assignment was carried out manually, the Agilent system has inbuilt software that carries out automatic feature extraction. Feature Extraction (FE) version 10.7 assigns grids and extracts information from probe features from microarray scan data, allowing measurement of miRNA. This software also contains QC metrics, which carries out quality control on each sample and generates QC reports for all samples and a project run summary.

5.2.5.4 Agilent data analysis and results

Data from our samples generated by the FE software was loaded into the “R” statistical software. Dr. John Pearson, Consultant Biostatistician with the University
of Otago, Christchurch, analyzed the data. Multiple testing greatly increases the chances of declaring false significance so to account for this adjusted $P$-values were calculated using Bonferroni and Sidak adjustments from the raw $P$-values.

We compared the samples under various headings including histologic subtype, PRETEXT stage, relapse, IHC markers and $CTNNB1$ mutation status and looked for miRNA expression patterns that could discern between subgroups with statistical significance. Comparison of pure fetal and mixed fetal/embryonal subtypes did not reveal any significant differences in miRNA expression. Nor did comparison of relapse versus non-relapse patients. Analysis of PRETEXT staging revealed that miR-100 was highly expressed in the four PRETEXT-3 tumours and not expressed in at all in PRETEXT-2 tumours. MiR-100 overexpression is believed to be involved in mTOR-related drug resistance and its overexpression has also been reported in medulloblastoma, prostate, nasopharangeal and ovarian cancers (Shi et al., Nagaraja et al., Leite et al., 2009, Liu et al., 2009).

Analysis of samples with $CTTNB1$ mutations versus wild-type identified a four miRNA signature that differentiates between the subgroups with statistical significance (Table 5.5.). MiR-342-3p, miR-20b, miR-223 and miR-99a were all significantly overexpressed in mutant versus WT. MiR-20b over-expression has been associated with decreased survival in gastric cancers and lymphoma (Di Lisio et al., Guo et al., 2009) and has a role in adaptation to oxygen concentration in tumours by regulating HIF-1 and VEGF (Lei et al., 2009). Overexpression of miR-223 has been demonstrated in many cancers including colon cancer, gastric cancer and lung cancer (Earle et al., Li et al., Miko et al., 2009).

Table 5.5: The four miRNAs differentially expressed between tumours with $CTNNB1$ mutations versus wild-type $CTNNB1$.

<table>
<thead>
<tr>
<th>ID</th>
<th>logFC</th>
<th>AveExpr</th>
<th>P.Value</th>
<th>adj.P.Val</th>
<th>mutant</th>
<th>WT</th>
</tr>
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<tr>
<td>hsa-miR-342-3p</td>
<td>7.91</td>
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<td>3.28E-05</td>
<td>0.0075</td>
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<td>0</td>
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<tr>
<td>hsa-miR-20b</td>
<td>6.48</td>
<td>4.01</td>
<td>5.66E-05</td>
<td>0.0075</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>hsa-miR-223</td>
<td>8.39</td>
<td>5.40</td>
<td>1.48E-04</td>
<td>0.0131</td>
<td>5</td>
<td>0</td>
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<tr>
<td>hsa-miR-99a</td>
<td>7.55</td>
<td>4.69</td>
<td>3.17E-04</td>
<td>0.0211</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>
5.2.5.5 Validation of array results

In order to test our hypothesis that there was a different miRNA signature in *CTNNB1* mutated and WT hepatoblastoma samples we arrayed a further sixteen samples from our cohort. We chose the samples on the basis of their mutation status and these were the eight remaining samples with detectable mutations and eight WT samples. To maximize the information from the sixteen samples we ensured an even mix of relapse and non-relapse patients in the two groups. This allowed us to carry out a secondary analysis of relapse *versus* non-relapse in a larger group of patients.

Table 5.6: Clinical and histologic characteristics of HB patients used in miRNA validation arrays

<table>
<thead>
<tr>
<th>CCRG</th>
<th>Type</th>
<th>Subtype</th>
<th>Pretext</th>
<th>EFS</th>
<th>CTNNB1</th>
<th>CyclinD1</th>
<th>Y654</th>
</tr>
</thead>
<tbody>
<tr>
<td>9T</td>
<td>epithelial</td>
<td>fetal</td>
<td>2</td>
<td>1</td>
<td>305a&gt;M</td>
<td>neg</td>
<td>neg</td>
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<td>314t&gt;K</td>
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<td>cyto</td>
</tr>
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<td>1</td>
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<td>cyto + nuc</td>
</tr>
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<td>0</td>
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<td>cyto</td>
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<td>0</td>
<td>310g&gt;R</td>
<td>neg</td>
<td>cyto</td>
</tr>
<tr>
<td>63T</td>
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<td>mixed</td>
<td>2</td>
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<td>304g&gt;R</td>
<td>neg</td>
<td>cyto</td>
</tr>
<tr>
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<td>epithelial</td>
<td>fetal</td>
<td>3</td>
<td>0</td>
<td>311g&gt;R</td>
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<td>cyto</td>
</tr>
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</tr>
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<td>1</td>
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<td>cyto</td>
</tr>
<tr>
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<td>macrotrab</td>
<td>2</td>
<td>0</td>
<td>WT</td>
<td>pos</td>
<td>cyto + nuc</td>
</tr>
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<td>epith/mesench</td>
<td>fetal</td>
<td>3</td>
<td>0</td>
<td>WT</td>
<td>neg</td>
<td>cyto</td>
</tr>
<tr>
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<td>fetal</td>
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<td>0</td>
<td>WT</td>
<td>neg</td>
<td>cyto + nuc</td>
</tr>
<tr>
<td>36bT</td>
<td>epithelial</td>
<td>fetal</td>
<td>3</td>
<td>0</td>
<td>WT</td>
<td>pos</td>
<td>cyto</td>
</tr>
</tbody>
</table>

The resulting array data was analysed as before using the R statistical package and the same biological and clinical comparisons made. All significance was lost when analysis was carried out on the larger independent cohort showing that the initial results were purely due to chance (adjusted *P*-values > 0.05). The only differentially expressed miRNA with an adjusted *P*-value approaching significance (adj. *P*-value = 0.054) was miR-20b, which appeared to be up-regulated in HBs with heterogenous expression of E-cadherin compared with normal membranous expression.
Figure 5.9: Heatmap generated by hierarchical clustering analysis
5.2.5.6 Hierarchical clustering analysis of miRNAs in HB

Unsupervised clustering analysis was carried out using Genesis software (Sturn et al., 2002). This is a technique used to identify patterns inherent in a data set. In the context of miRNA arrays it recognises “clusters” of miRNAs with similar expression patterns. Figure 5.10 shows the heatmap generated from unsupervised clustering of our data. Only the 180 miRNAs expressed in one or more of the tumour samples are shown. The remaining human 686 human miRNAs were not detected in any of the tumour sample using Agilent microarrays. The samples are shown across the top with the dendrograms along the left-hand side. The longer the branches on the dendrograms, the more tightly the miRNAs are co-expressed. MiRNAs that are co-regulated, are involved in the same pathways or are from the same tissue are likely to be clustered. Very little evidence of clustering was found from miRNA analysis of our sample cohort. From this we can assume that there is little differential expression of miRNAs between tumours with different biologic profiles.

5.2.5.7 Differentially expressed miRNAs between HB and normal liver

Several studies have been carried out to determine the miRNA expression profile of liver tissue. A meta-analysis of these studies reported 92 miRNAs expressed in adult and/or fetal liver as shown in Table 5.1. We analysed our expression profiles to identify miRNAs differentially expressed with regard to normal liver expression profiles. Of the 180 miRNAs expressed in HB tumours, 76 have been previously reported in normal liver tissue as shown in Table 5. The sixteen highlighted rows represent miRNAs whose expression is absent from our entire cohort of twenty-three HB samples.

Further investigation of miRNAs down-regulated in our tumours samples compared with published liver miRNA profiles revealed that miR-378, which is absent from our HB samples, is down-regulated following hepatectomy and this directly promotes hepatic regeneration through DNA synthesis (Song et al.). Repression of miR-378 in hepatoblastoma may allow for proliferation of tumour cells by the same mechanism. MiR-186, down-regulated in our tumour set, was up-regulated in a study of HCC (Wang et al., 2008).
Table 5.7: Published Liver miRNA data with corresponding HB miRNA expression data from this study. Highlighted rows indicate miRNAs not expressed in any HB tumour analysed.

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One hundred and eight miRNAs expressed in one or more of our samples have not previously been reported in normal liver tissues. We further analysed these miRNAs and identified those that are expressed in ten or more HB samples with a fold-change greater than 1.2. This identified sixty-two miRNAs as shown in Table 5.8. A review of the literature revealed that up-regulation of eighteen of these miRNAs has previously been reported in hepatocellular carcinoma. Interestingly, three of these miRNAs, hsa-miR-200b, hsa-miR-214 and hsa-miR-215 were shown to be down-regulated in studies of HCC (Gramantieri et al., 2007, Wang et al., 2008). Forty-one miRNAs expressed in hepatoblastoma have not previously been reported in hepatic cancer.

Table 5.8: miRNAs up-regulated in HB

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Green shaded indicates down-regulated in HCC and orange shaded indicates up-regulated in HCC.
5.3 Discussion

In the course of this study the field of microRNA research has greatly expanded. The current number of miRNAs is nearly three times the number reported in 2006. The technology used to study miRNAs has also developed to become fast, reliable and very sensitive in the intervening time. Numerous array-based studies have been performed using a variety of different tumour and non-tumour tissues. The current hurdle remains the validation of miRNA array results and the annotation of miRNAs. While web-based target prediction software is available and may indicate target mRNA and pathways regulated by particular miRNAs, only a fraction have been validated in biologic systems.

The importance of validating array findings in an independent data set has been highlighted in our study. Our initial Agilent miRNA array data pointed to a differential expression pattern in mutant and wild type CTNNB1 tumours. All significant differential expression of miRNAs between the two groups was lost when we carried out analysis on a larger cohort of tumour samples. Analysis of biologic subgroups in our cohort revealed only a single differentially expressed miRNA, miR-20b, which appeared to be up-regulated in HBs with heterogenous expression of E-cadherin compared with normal membranous expression. Heterogenous expression of E-cadherin implies a loss of normal membranous expression in a proportion of tumour cells within a sample and this E-cadherin loss is associated with loss of cellular adhesion and a more invasive phenotype. MiR-20b has been reported as being associated with cancer progression in a variety of cancers but never with a loss of E-cadherin or increased EMT to date.

Analysis of miRNA expression profiles of twenty-three hepatoblastoma samples revealed a large overlap with miRNA expression in normal adult and fetal liver. Of ninety-two miRNAs reported in normal liver, seventy-six were expressed in HB tumours. Lack of expression of sixteen miRNAs in all HB samples may indicate miRNAs with potential tumour suppressor function. One of these miRNAs, miR-192 has been shown to act as a tumour suppressor by inducing cell cycle arrest (Braun et al., 2008). Recently miR-192 has also been shown to positively regulate levels of E-
cadherin at the cell membrane. As a result, down-regulation of miR-192 may potentially contribute to EMT. Down-regulation of miR-378 has been demonstrated following hepatectomy and this is thought to promote DNA synthesis in hepatocytes and contribute to hepatic regeneration (Song et al.). Down-regulation of miR-378 seen in our HB tumour samples may have a similar effect and promote tumour development. Down-regulation of miR-148a was reported in a study of HB by Magrelli et al but this was not replicated in our HB cohort (Magrelli et al., 2009).

Sixteen miRNAs highly expressed in HB tumour samples have previously been reported in HCC studies (Varnholt, 2008). These include miRNAs with known oncogenic function such as miR-106b that promotes cell cycle progression by regulating p21 and CDKN1A (Ivanovska et al., 2008) and miR-10b that regulates HoxD10 (Ma et al., 2007). Interestingly, miR-214, an oncomir shown to induce cell survival and cisplatin resistance by targeting PTEN (Yang et al., 2008), was also up-regulated in our HB samples but down-regulated in HCC (Wang et al., 2008). We uncovered forty-one miRNAs previously unreported in hepatic tumours. Many of these are recently discovered miRNAs with no known function to date. Although miRNA research holds much promise for clinical diagnostics and potential therapeutics, the challenge for future research will be to elucidate specific biologic roles for these miRNAs.
Chapter Six

Discussion and Future Directions
Chapter 6: Discussion and Future Directions

6.1 Discussion

The majority of childhood cancers are sporadic, not inherited, and as they present at such a young age are unlikely to have environmental causes. Because it is not possible to prevent embryonal tumours of Table 1.1: Current classification of HB

Table 1.2 Definitions of PRETEXT number

Table 1.3 Risk stratification criteria for SIOP-EL

Table 1.4: Traditional COG staging system, childhood, the emphasis must be on effective treatment with stage and resection margins, minimal side effects to allow affected children to recover and lead normal healthy lives. Although the survival rates for hepatoblastoma patients have improved dramatically in the past 30 years with the advent of adjuvant chemotherapy, there still remain around 30% of patients who die from the disease. These are mainly patients who are classified as high risk at diagnosis although disease progression also occurs in some 10% of standard risk patients.

Table 2.1: A list of centres participating in this hepatoblastoma study

Table 2.2: Adult liver control samples

Table 2.3 Antibodies used in Western blot analysis showing working dilutions and antibody species

Table 2.4: Scan settings used for miRNA array scanning

Table 4.1 Histologic type and subtype, β-catenin and Y654 β-catenin IHC and CTNNB1 gene status of hepatoblastomas with mutations

Table 4.2 Review of published β-catenin mutation/expression studies

Table 5.1: Repertoire of miRNAs in human liver samples used in miRNA array analysis showing RNA and miRNA concentrations

Table 5.2 Mouse liver samples used in miRNA array analysis showing RNA and miRNA concentrations

Table 5.3: The ten highest ranking miRNAs identified by array analysis

Table 5.4 Clinical and molecular characteristics of HB patients used in the initial Agilent miRNA microarray experiment
Table 5.: The four miRNAs differentially expressed between tumours with CTNNB1 mutations versus wild-type CTNNB1.

Table 5.: Clinical and histologic characteristics of HB patients used in miRNA validation.

Figure 1.2: β-catenin protein and mutations seen in cancer.

Figure 1.4: Schematic diagram of the epithelial-mesenchymal transition.

Figure 1.5: Biogenesis of miRNAs.

Figure 2.1: Schematic diagram of iBlot® Dry Blotting system showing the flow of current.

Figure 2.2: Schematic layout of an Agilent 8x15K miRNA array slide.

Figure 2.3: A QC Report for 8x15K miRNA microarray.

Figure 3.1: Schematic diagram showing the construction of a tissue microarray.

Figure 3.2: Example of a tissue microarray section used in this study.

Figure 3.3: Excel sheet layout of TMA-1A.

Figure 3.4: Excel sheet layout of TMA-1B.

Figure 3.5: Staining of normal adjacent liver tissue using an antibody to β-catenin.

Figure 3.6: Cytoplasmic localization of β-catenin.

Figure 3.7: Nuclear localization of β-catenin.

Figure 3.8: Survival curves for β-catenin protein expression.

Figure 3.9: Normal E-cadherin staining pattern.

Figure 3.10: Heterogenous E-cadherin expression.

Figure 3.11: Survival curves for E-cadherin expression.

Figure 3.12: Positive AFP staining.

Figure 3.13: Negative staining for AFP in hepatoblastoma.

Figure 3.14: Survival curve for AFP.

Figure 3.15: Staining of Cyclin D1 by IHC.

Figure 3.16: Cyclin D1 negative staining.

Figure 3.17: Survival curves for Cyclin D1.

Figure 3.18: High Ki67 score.

Figure 3.19: Low Ki67 score.

Figure 3.20: EFS survival curves for Ki-6.

Figure 4.: RNA quality measurements.

Figure 4.: Agarose gel showing amplified β-catenin RT-PCR products.

Figure 4.: Amplicons from samples 82 and 15 with point mutations in forward and reverse sequences highlighted in yellow.

Figure 4.6: Frequency of total β-catenin and Y654-β-catenin expression in our Hepatoblastoma cohort.

Figure 4.7: Direct sequence analysis of exon 3 of β-catenin in HuH-7 and HuH-6 cell lines.

Figure 4.8: BCA assay for nuclear proteins.

Figure 4.9: BCA assay for cytoplasmic proteins.

Figure 4.10: Western blot to detect total β-catenin levels after timecourse experiment.

Figure 4.11: Western blot to examine β-catenin and Y654-β-catenin expression.
In our biomarker study we sought to identify a tumour biomarker that may aid in prediction of relapse in SR patients. We examined the expression of five proteins in HB tumour samples by immunohistochemistry in a large cohort of patients enrolled on the SIOPEL-3 hepatoblastoma clinical trial. We then analyzed the results with respect to event-free and overall survival. We found that two of our markers, Ki-67 and CyclinD1, were significantly associated with EFS in our cohort. The Ki-67 protein is a cellular marker for proliferation and the fraction of Ki-67-positive tumour cells (the Ki-67 labelling index) is often correlated with the clinical course of cancer. A previous study in a small number of HB tumours (n = 14) determined that a Ki-67 labelling index >10 correlated with lowered survival from the disease (Ara et al., 1997), and our study validated these findings in a larger HB cohort (n = 71). CyclinD1 is a cell-cycle mediator and is a transcription target of β-catenin. Its over-expression has been linked to many cancers and our study found that CyclinD1 expression correlated with EFS in our cohort. This has identified two markers that could potentially be used to aid prognostication and identify standard risk patients that are at risk of relapse.

In addition to these findings we uncovered a novel correlation between the expression of CyclinD1 and tyrosine 654-phosphorylated β-catenin. Upon ligand binding of HGF to its receptor, c-Met, β-catenin becomes phosphorylated at specific tyrosine residues, including tyrosine 654. This prevents β-catenin degradation and leads to accumulation in the nucleus and activation of transcription targets. It has been presumed that the means of nuclear β-catenin accumulation (Wnt activation, c-Met activation or activating mutations) does not differentially affect its downstream transcription. However, we found a significant association of CyclinD1 with Y654-β-catenin but
not with expression of total β-catenin and this indicates that HGF/c-Met activation of β-catenin may lead to transcription of specific target genes.

We also examined any potential correlation with histologic type or subtype that might aid in classification of this complex tumour. With the exception of CyclinD1, which appears to be expressed more highly in mixed epithelial/mesenchymal HBs, our biomarkers were not highly informative with regard to histology. This may be a reflection on the very complex nature of the tumour. HBs often show very varied histology within a tumour sample, and differentiating between fetal and embryonal cells and between epithelial and mesenchymal elements demands a level of expertise beyond the scope of this study.

Although β-catenin expression did not correlate with histology or outcome in our cohort, aberrant expression was seen in 87% of our tumour samples. This is reflective of the findings of other hepatoblastoma studies (Curia et al., 2008, Koch et al., 2005, Park et al., 2001b). A wide variety of mutation frequencies of the β-catenin gene have been reported in HB. We amplified and sequenced a region of exon 3 of CTNNB1 to determine whether activating mutations of CTNNB1 may account for β-catenin accumulation in our cohort. We found that 14% of our tumour samples harboured mutations in GSK3β and a further 6% may have deletions of exon 3, although we were unable to confirm this. The mutations seen in our study have all been reported in previous HB studies. The mutation frequency of our cohort is one of the lowest when compared to all published reports. The disparity between the high levels of protein expression and low mutation frequency prompted us to investigate an alternative β-catenin activation pathway in HB.

β-catenin activation by HGF/c-Met signalling has been reported in several tumours including hepatoblastoma (Ranganathan et al., 2005b). c-Met is the tyrosine kinase receptor for hepatocyte growth factor (HGF), that upon ligand binding, triggers the activation of several pathways controlling epithelial-mesenchymal morphogenesis, angiogenesis and cell-cell adhesion, including β-catenin (Zeng et al., 2006a). In the liver, the HGF/c-Met pathway has a crucial role in the activation of liver cell regeneration following injury or partial heptectomy (Peruzzi and Bottaro, 2006). Our
study found that nuclear accumulation of β-catenin in ~30% of our HB tumours was due to c-Met activation. Our novel findings also showed that c-Met activation of β-catenin occurs distinctly from tumours with activating β-catenin mutations (Purcell et al, submitted). These findings identify a large subset of HB that would be susceptible to receptor tyrosine kinase (RTK)-targeting agents. Several of these types of small-molecule c-Met-inhibitors are now in early phase clinical trials in adult cancers (Bagai et al., Liu et al.).

In addition to the therapeutic potential, our findings of high levels of c-Met activated β-catenin may shed some light on the underlying biology of the tumour. The incidence of hepatoblastoma has been on the rise in the last thirty years and this is thought to be due to the increased survival of preterm infants. Although prematurity itself is not linked with increased risk of developing HB, many preterm infants have very low birth weight (VLBW), and VLBW babies (<1,000g) are fifteen times more likely to develop HB than babies >2,500g (Herzog et al., 2000). Similar findings were reported more recently (Slovis and Roebuck, 2006, Spector et al., 2008) and interestingly children with low birth weight tend to have a later age-of-onset of HB (Reynolds et al., 2004). The reason why LBW babies are at increased risk of HB has not been widely explored although increased exposure to steroids and mechanical ventilation have been postulated.

We propose that HB in some part may be caused by increased levels of HGF produced during “catch-up” growth in LBW infants. Catch-up growth is a phenomenon observed in LBW babies who undergo an accelerated period of growth usually between two months and two years of age (Monset-Couchard and de Bethmann, 2000). This catch-up growth has been associated with insulin-resistance in later life but a link with cancer development has not been investigated. The liver is still maturing in the perinatal period and we propose that after a period of growth restriction in LBW infants, an increased level of HGF during the catch-up growth phase may contribute to the development of HB through activation of β-catenin. To date, no studies have measured levels of serum HGF in LBW babies but one study in sheep provides some anecdotal evidence for our theory. This study found that sheep born to nulliparous mothers, displayed catch-up growth by the first month of life and
that these offspring expressed significantly higher levels of HGF mRNA than matched controls (Hyatt et al., 2007). The role of HGF/c-Met activation of β-catenin in HB pathogenesis represents an exciting research prospect for the future.

Expression profiling using micro-array technology has identified miRNA patterns associated with different tumour types (Volinia et al., 2006) and the current hurdle is to define roles for the individual miRNAs associated with cancer. The majority of miRNAs are believed to act as tumour suppressors and are down-regulated as the tumour becomes less differentiated (Gregory et al., 2008). In the final part of this study we examined miRNA profiles from twenty-three HB tumours and identified sixteen miRNAs whose expression is lost compared with normal liver tissue, including miR-192, which has known tumour suppressor function. Our analysis also revealed a large number of miRNAs over-expressed in HB. These include sixteen miRNAs also over-expressed in HCC and an additional forty-one miRNAs not previously reported in hepatic tumours. Future work will aim to prospectively validate and define roles for these miRNAs and identify specific miRNAs involved in chemotherapeutic resistance. As miRNAs are occur naturally in the cell, they are unlikely to have “off-target” effects and may result in much lowered toxicity and present a very exciting therapeutic prospect.

The translation of our findings may be important for design of future clinical trials, identifying patients for individual targeted therapy and allowing for fewer side effects. Tumours such as medulloblastoma and neuroblastoma display activation of many of the same signalling pathways as hepatoblastoma, including Wnt/β-catenin. Therefore, our findings may also have a wider application in the understanding and treatment of other embryonal tumours of childhood.

6.2 Future Directions

6.2.1 To elucidate the role of HGF/c-Met signalling in HB pathogenesis.
In future studies HB cell lines, HuH-6, HepG2 and Hep293TT, will be used to investigate the effect of HGF/c-Met activation on cell proliferation, migration, invasion and epithelial-to-mesenchymal transition. The cell lines will be grown in the presence/absence of HGF and use the xCELLigence system (Roche Diagnostics) to
determine cell viability, proliferation, invasion and migration. The relative levels of specific targets of β-catenin e.g. CyclinD1 and Survivin will also be assessed after activation of the HGF/c-Met pathway. Several different HGF inhibitors and TRK inhibitors will be applied and the cellular responses listed above will be monitored. These experiments will determine efficacy and dose-response in HB cell lines.

6.2.2 To demonstrate specific interactions between miRNAs and their mRNA targets in vitro, and validate these findings in our HB cohort.
Validation of miRNAs found to be highly expressed or with decreased expression will be carried out on the entire HB cohort (n=94) and examine the molecular roles of these individual miRNAs in HB cell lines. Based on these results it will be possible to determine the effect of re-introduction of tumour suppressor miRNAs and the possible value in reactivating pathways that drive a therapeutic response. Using the same cell lines as previously specific pre-miRNAs, found to be underexpressed in HB by array analysis will be transfected to examine the effect on proliferation, invasion and migration of the cells and the putative mRNA targets assayed using qPCR and western blot. Conversely overexpressed miRNAs will be knocked-down using miRNA inhibitors and the findings validated on the HB-TMA using miRNA in situ amplification and co-detection of mRNA targets.

6.2.3 To determine whether miRNA deregulation in HB is controlled by copy-number changes in the genome.
The means by which miRNAs themselves are regulated has not been intensely studied to date. Several papers have reported epigenetic regulation of miRNA genes by methylation at the DNA level. Array Comparative Genomic Hybridization (aCGH) will be carried out on DNA samples from corresponding HB tumours and adjacent normal tissue. Standard CGH has reported various chromosomal aberrations in HB but aCGH will allow us to detect microdeletions and copy number changes that will be correlated with miRNA expression profiles to determine whether miRNA expression may be regulated at this level. This is a novel concept and if successful may be useful in understanding the complex regulation of cancer.
In summary, hepatoblastoma is a complex tumour that warrants further investigation into the molecular pathology of the disease. Understanding the underlying biology is necessary for developing more targeted therapy to improve survival. Elucidating the molecular mechanisms of HB may also provide insight into other paediatric neoplasms and adult cancers that share similar biologic activation. In this study, archival paraffin-embedded tumour samples were accessed and successfully used to carry out protein and nucleic acid analyses. This type of sample is an under-utilized resource that could be successfully used in future cancer studies. This study accessed a relatively large number of samples of this rare tumour but a much larger cohort would be needed to confirm the biologic notions proposed in this study, underlying the need for further international collaboration.
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