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Differential Expression of TWSG1, BMP4 and Shh Morphogens Signaling Proteins in Hepatocellular Carcinoma and Cholangiocellular Carcinoma

by

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I dedicate this thesis to my beloved family for all their love and support

Abstract

Hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCA) constitute two of the most common liver malignancies in adults. The molecular mechanisms underlying their development remain poorly understood.

Morphogen proteins, including the hedgehog and the bone morphogenetic proteins pathway fulfill a major role in embryonic development. Consequently, alterations in these proteins may cause embryonic defects. Furthermore, abnormal expression of morphogen proteins is found to correlate with cancer development and progression.

This thesis has demonstrated the differential expression and co-localization of the morphogen proteins, including sonic hedgehog (Shh), bone morphogenetic protein (BMP)-4 and twisted gastrulation protein (TWSG)-1 in human HCC and CCA tumors and cell lines. These proteins are strongly expressed in CCA more than in HCC.

Overall, these findings suggest that morphogen proteins may play a crucial role during liver carcinogenesis and progression, specifically in CCA. Moreover, these proteins can serve as diagnostic biomarkers for HCC and CCA.

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List of abbreviations

ABC	Avidin biotin complex
AFP	Alpha-fetoprotein
ALP	Alkaline phosphatase
Alpha-antitrypsin	Alpha-antitrypsin
ATP	Adenosine triphosphate
BAMBI	BMP and activin membrane-bound inhibitor
Bcl-2	B-cell lymphoma 2
BMP	Bone morphogenetic proteins
BMPR	Bone morphogenetic protein receptor
C. sinensis	*Clonorchis sinensis*
CA 19-9	Carbohydrate antigen 19-9
CA-125	Carbohydrate antigen-125
CCA	Cholangiocellular carcinoma
CD34	Cluster of differentiation 34

CEA	Carcinoembryonic antigen
CK	Cytokeratin
CO2	Carbon dioxide
CT	Computerized axial tomography
DAB	Diaminobenzidine
Dhh	Desert hedgehog
DNA	Deoxyribonucleic acid
E.	Embryonic day
EMT	Epithelial mesenchymal transition
FBS	Fetal Bovine Serum
FFPE	Formalin fixed paraffin embedded tissue
FGF	Fibroblast growth factors
Fu	Fused
G1	Grade 1 (well-differentiated tumor)
G2	Grade 2 (moderately-differentiated tumor)

G3	Grade 3 (poorly-differentiated tumor)
G4	Grade 4 (undifferentiated tumor)
Gamma-GTP	Gamma glutamyl transpeptydase
GIT	Gastrointestinal tract
Gli	Glioma-associated oncogene family zinc finger 1
GPC3	Glypican-3
H-Ras	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
H&E	Hematoxylin and eosin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
Hep Par 1	Hepatocyte paraffin 1
HGF	Hepatocytes growth factor
Hh	Hedgehog

HNF-4α	Hepatocyte nuclear factor 4 alpha
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
Ihh	Indian Hedgehog
IL6	Interleukin-6
Le^a	Lewis antigen ^{a}
Le^b	Lewis antigen ^{b}
M6P/IGFR-II	Mannose-6-phosphate/Insuline growth factor receptor
Mcl-1	Induced myeloid leukemia cell differentiation protein-1
MOC 31	Monoclonal antibody 31
MRI	Magnetic resonance imaging
MTA1	Metastasis-associated protein
NF-kB	Nuclear factor kappa B
O. viverrini	*Opisthorchis viverrini*

p-CEA	Polyclonal Carcinoembryonic antigen
PDGFR	Platelet-derived growth factor receptor
PSC	Primary sclerosis cholangitis
Ptch	Patch
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shh	Sonic Hedgehog
Ski	Sloan–Kettering retrovirus
SMAD	Small Mothers against decapentaplegic protein
SMADR	Small Mothers against decapentaplegic protein receptor
SMO	Smoothend
Sog	Short gastrulation
SOX9	SRY (sex determining region Y)-box 9

STM	Septum transversum mesenchyme
Sufu	Suppressor of fused
SV40 T	Simian virus 40 large T antigen
TGF-β	Transforming growth factor beta
TGF-βR	Transforming growth factor beta receptor
ThO2	Thorium dioxide
TMA	Tissue microarrays
TNF- α	Tumor necrosis factors-alpha
TP53	Tumor protein 53
TTBS	Tris-Buffered Saline and Tween 20
TWSG	Twisted gasturlation protein
USA	United States of America
VIP	Violet reaction products
WHO	World health organization

Chapter 1

Literature review

1.0 Overview

Cancer is a malignant form of uncontrolled growth and spread of cells in the mammalian body. Liver cancer is the fifth most common fatal cancer worldwide (Parkin *et al*., 2005). Hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCA) are the most common types of liver cancer reported worldwide. Case reports and studies suggest that the increased incidence of liver cancers (HCC and CCA) is due to the lack knowledge of the molecular mechanisms, pathogenesis and the etiological factors (Bosch *et al*., 2004). Furthermore, liver cancer is rarely diagnosed at the early stage; hence it is known as the "silent killer", and often diagnosed at an advanced stage (Motola-Kuba *et al*., 2006; Khan *et al*., 2005; Olnes and Erlich, 2004).

Tumor markers are very important diagnostic tools, used in the early detection of cancer to determine the prognosis ("the survival rate") of patients, to assess the response to therapy in patients and to monitor cancer recurrence.

Morphogens are proteins that play essential roles during embryonic development and are found to be reactivated during carcinogenesis, making them useful in cancer diagnosis (Gekas *et al*., 2012; Hahn *et al*., 1996). Sonic hedgehog (Shh),

bone morphogenetic protein (BMP)-4 and their components are examples of the morphogen proteins. Alteration or abnormal expression level of these proteins is related to cancer development (Hahn *et al*., 1996; Guo *et al*., 2012).

This chapter will discuss the role of several morphogenetic proteins including Shh, BMP4 and twisted gastrulation protein 1 (TWSG1) during embryonic development and tumor development. This chapter will also provide a brief overview in human liver anatomy, embryonic liver development including the differentiation of liver cells and bile duct cells. Finally, in this chapter There will be a brief overview on liver carcinogenesis including HCC and CCA including their epidemiology, risk factors, pathogenesis, clinical and laboratory diagnosis, treatment and liver cell lines.

1.1 Embryonic morphogen signaling pathways

Early embryonic development involves several cellular activities, including cellular proliferation and differentiation. These processes are involved in some temporal patterns of cell and tissue development (Hartenstein, 1989; Megason and McMahon, 2002). In particular, part of this patterning process entails the concentration gradient of morphogens. Wolpert proposed the term “morphogens” in 1969 to describe specific secreted signaling molecules that act on cells to initiate cell fate through a concentration-dependent manner (Figure 1.1) (Wolpert, 1969; Tabata and Takei, 2004). Few proteins act as morphogens and include hedgehog (Hh) proteins (Pierani *et al*., 1999) and transforming growth factor-beta

(TGF-β) proteins (Lecuit and Cohen, 1998). These molecules form concentration gradients to specify the cell fate during organogenesis in a concentration-dependent manner. As these molecules fulfill an essential role during embryonic development and organogenesis, several studies have demonstrated their functional role in carcinogenesis (Liao *et al*., 2009; Nhieu *et al*., 1999; Rich *et al*., 2001).

1.1.1 The Hedgehog signaling pathway

The Hedgehog (Hh) gene was named according to a phenotype during a mutagenesis screen conducted by two drosophila geneticists, Christiane Nusslein-Volhard and Eric Wieschaus (Nusslein-Volhard and Wieschaus, 1980). Specifically, when the Hh function is lost by knockout or mutation, the mutant larva grows small projections covering its entire surface, which possesses the appearance of a hedgehog. Hh performs a central role during development, controlling segmentation patterning of the drosophila (Nusslein-Volhard and Wieschaus, 1980; Merchant, 2012). Moreover, Hh was found to fulfill crucial functions during mammalian development, promoting mitogen-mediated cell proliferation, cell survival, and tissue polarity, as well as acting as a morpohgen mediating cell fate in a dose-dependent manner (Bhardwaj *et al*., 2001; Riddle *et al*., 1993; Torroja *et al*., 2005; Yu *et al*., 2002) Furthermore, studies have shown that Hh signaling is paramount to foregut development, including the liver (Hirose *et al*., 2009; Litingtung *et al*., 1998).

The mammalian hedgehog family consists of three secreted ligands: 1-sonic hedgehog (Shh), 2-indian hedgehog (Ihh) and desert hedgehog (Dhh) (Kumar *et al*., 1996; Pathi *et al*., 2001). All three secreted proteins bind to their receptor with a similar affinity; however, their expression patterns vary depending on their potency and tissue specificity (Pathi *et al*., 2001). Specifically, Shh expression is strongly expressed in the embryonic foregut (Litingtung *et al*., 1998). On the other hand, Ihh expression is strongly expressed in the embryonic hindgut (Kolterud *et al*., 2009). The three proteins bind to their receptor, patched (Ptch)-1, a 12-transmembrane receptor that inhibits the 7-transmembrane protein signaling receptor smoothened (SMO) (Dessaud *et al*., 2008). Absence of the Hh ligand causes the Shh signaling pathway to become inactivated (Dessaud *et al*., 2008). In contrast, the presence of the Shh ligand, which binds to its receptor Ptch-1, subsequently releases an inhibitory effect on SMO. SMO then releases the inhibition of the proteins complex of fused (Fu) and suppressor of fused (Sufu), on glioma-associated oncogene (Gli). The uncleaved Gli enters the nucleus and targets Hh genes (Figure 1.2) and other genes, including platelet-derived growth factor receptor (PDGFR), Cyclin D1 and bone morphogenetic proteins (BMPs) (Dessaud *et al*., 2008; Huang *et al*., 2011; Kawai and Sugiura, 2001; Shahi *et al*., 2010).

Shh, a secreted glycoprotein, regulates the anterior/posterior patterning in limb development, the polarity in the central nervous system, including the ventral portion of neural tube formation (Marigo and Tabin, 1996), and many other cell-type differentiations (Levine *et al*., 1997; Yu *et al*., 2002; Cao *et al*., 2010).

During embryogenesis, the dysregulation or mutations in the Shh signaling pathway has been reported to cause embryonic developmental defects, such as holoprosencephaly, a cephalic disorder in which the prosencephalon fails to develop into two hemispheres during brain embryonic development (Gekas *et al*., 2012). However, the upregulation of Shh and/or mutations in the tumor suppressor ptch-1 and the proto-oncoprotein SMO have been found to fulfill an important role during carcinogenesis (Hahn *et al*., 1996; Reifenberger *et al*., 1998; Yoshikawa *et al*., 2009).

1.1.2 Transforming Growth Factor

TGF-β (transforming growth factor-beta) is a superfamily of polypeptide growth factors including the TGF-β1, 2, 3 superfamily (Piek *et al*., 1999). TGF-β is one of the major regulators in embryonic development, including liver development, controls cell proliferation, cell differentiation and cell death (Shen, 2007; Zorn, 2008). The inhibition of TGF-β was identified as playing a critical role in hepatocarcinogenesis and cell proliferation in many cancers including liver cancer (Marotta *et al*., 2004).

Two TGF-β receptors have been identified: TGF-βR-I and TGF-βR-II, each contain an extracellular binding domain and intracellular serine/threonine kinase domains. A third TGF-β receptor (TGF-βR-III) does not have intracellular signaling; however, it mediates TGF-βR-I and TGF-βR-II interactions (Yingling *et al*., 1995). TGF-β is activated and binds to TGF-βR-II in response to mannose-

6-phosphate/insulin growth factor receptor-II (M6P/IGFR-II) and IGF-RII. This process, in turn, leads to the transphosphorylation of TGF-βR-I. Afterwards, the transcription of the small mothers against decapentaplegic homolog (SMAD) receptors (SMAD2-R and SMAD3-R) is triggered, forming a complex, which is activated via phosphorylation. Phosphorylated SMAD receptors bind with SMAD4, forming a complex that translocates into the nucleus and activates the transcription of the target genes (Rich *et al*., 2001).

TGF-β overexpression has been detected in 40% of patients with hepatocellular carcinoma (HCC) tumors, and their serum (Abou-Shady *et al*., 1999; Yuen *et al*., 2002). However, down-regulation of TGF-β receptors was reported in more than 50% of HCC cases (Sue *et al*., 1995). The correlation between median survival rate and the expression of TGF-β in patients with HCC, suggests that TGF-β can be used as a serological marker for HCC diagnosis (Song *et al*., 2002). However, the controversial issue resides behind the function of TGF-β receptor where it is suggested to be anti-proliferative by some studies (Gotzmann *et al*., 2002; Kang *et al*., 2003), but pro-proliferative by others (Moustakas and Heldin, 2007; Reichl *et al*., 2012). Interestingly, Gotzmann *et al*. confirmed that TGF-β inhibits cell growth and induces cell death in immortalized HCC cell lines (Gotzmann *et al*., 2002). Gotzmann added that the morphology of hepatocytes changes due to the activity of TGF-β, which regulates the transition of the polarized epithelial cells to a spindle shape. This process leads to the destruction of intercellular complexes at the final stages of cell death. Such morphological changes of hepatocytes may

occur in cooperation with the activated oncogene v-Ha-ras Harvey rat sarcoma viral oncogene homolog (H-Ras) enhancing proliferation and tumor progression (Gotzmann *et al*., 2002).

1.1.2.1 Bone morphogenetic proteins signaling pathway

Bone morphogenetic proteins (BMPs) are glycosylated extracellular matrix-associated molecules and members of the TGF-β superfamily (Walsh *et al*., 2010). In 1965, Urist isolated BMP from demineralized rabbit cortical bone (Urist, 1965; Urist and Strates, 1971). Subsequently, BMPs were identified as one of the central pathways that were involved in embryonic development, especially during the dorsal/ventral-patterning stage (Figure 1.1) (Liem *et al*., 1995). In contrast, BMPs were also identified in several vital cellular processes of adults, including cell proliferation and differentiation during diseases such as carcinogenesis (Luo *et al*., 2008). The large precursor monomer of BMP is cleaved by an extracellular protease, dimerized, and secreted outside of the cell (Chen *et al*., 2004; Walsh *et al*., 2010). Subsequently, the mature BMP dimers bind to their transmembrane receptors, BMPR-I and BMPR-II (Figure 1.3). BMPR-II activates the serine/threonine kinase of BMPR-I, which, in turn, triggers the intracellular signaling of the SMAD1/5/8 phosphorylation (Chen *et al*., 2004; Walsh *et al*., 2010). This phosphorylation eventually translocates SMAD4 from the cytoplasm to the nucleus, acting as a transcription factor that regulates the transcription of target genes (Chen *et al*., 2004; Walsh *et al*., 2010).

More than 30 proteins related to BMPs have been identified and sub-classified according to their functions and structures. Among these proteins, BMP4 is the most studied member of the BMP family (Ducy and Karsenty, 2000). BMP4 is a glycosylated extracellular protein that regulates the anterior/posterior patterning in limb development; the polarity in the central nervous system, including the ventral part of neural tube formation; and many other types of cell differentiation. The dysregulation or loss of the BMP4 signaling pathway during embryogenesis has been reported to cause embryonic lethality and/or developmental defects (Kaplan *et al*., 2006; Winnier *et al*., 1995). However, its role in adult diseases is controversial and poorly understood, especially in relation to carcinogenesis. Several studies have proposed that BMP4 enhances cellular proliferation and tumor growth via epithelial mesenchymal transition (EMT) (Guo *et al*., 2012; Lorente-Trigos *et al*., 2010). EMT is a biological process where the epithelial cell loses its polarity and cell-cell adhesion molecules, and transitions to migratory and invasive functions of mesenchyme cells (Kalluri and Weinberg, 2009). In contrast, other research has proposed that BMP4 acts as a tumor suppressor, inhibiting the progression and growth of tumors (Shirai *et al*., 2011). Alarmo *et al*. investigated the role of BMP4 expression in 34 different organs, including 486 breast cancer tissue samples (Alarmo *et al*., 2013). These authors suggested that the role of BMP4 role varies according to tumor type, and that the strong granular expression of BMP4 is correlated with low proliferation during the first step of tumor development and can subsequently stimulate cancer recurrence and metastasis (Alarmo *et al*., 2013).

1.1.2.2 BMP inhibitors

The binding of BMP ligands to their receptors is closely mediated by a large group of intracellular and secreted extracellular protein antagonists. The intracellular inhibition of BMPs involves SMAD inhibitors, such as SMAD-6 and 7 (Imamura *et al*., 1997; Nakao *et al*., 1997); intracellular SMAD binding proteins, such as Sloan–Kettering retrovirus (Ski) and transducer of Erb B-2 (Tob) (Wang *et al*., 2000; Yoshida *et al*., 2000); and SMAD ubiquitination regulatory factor (Smurf) 1 and Smurf 2 (Zhang *et al*., 2001; Zhu *et al*., 1999). Secreted extracellular inhibitors of BMPs include noggin (Groppe *et al*., 2002), chordin (Piccolo *et al*., 1996), follistatin (Fainsod *et al*., 1997) and twisted gastrulation (TWSG1) protein (Ross *et al*., 2001), as well as non-signaling pseudo-receptors, such as BMP and activin membrane-bound inhibitor (BAMBI) (Figure 1.3) (Onichtchouk *et al*., 1999). Studies have revealed that BMP inhibition is essential during embryonic development, and alterations in these molecules lead to major developmental abnormalities (Bachiller *et al*., 2000; Zakin and De Robertis, 2004). Nevertheless, there is some evidence that BMP inhibition can be associated with some diseases, including cancer (Moll *et al*., 2006; Pils *et al*., 2010).

1.1.2.2.1 Twisted gastrulation protein 1

Twisted gastrulation protein (TWSG)-1 was identified and named by Zusman *et al*. during the gastrulation period (a biological process where the monolayer of cells transform into the three layers ectoderm, mesoderm and endoderm) phenotype of the drosophila (Zusman and Wieschaus, 1985). TWSG1 was established as one of the BMP antagonists (Ross *et al*., 2001). Studies have shown

that TWSG1 performs an essential role during embryonic development; consequently, a defect in this protein may lead to an abnormality in craniofacial development (Petryk *et al*., 2004). The binding of TWSG1 and BMPs possesses unique characteristics, which can both enable and prevent BMP activities. TWSG1 is an extracellular secreted protein that inhibits the signaling activity of BMP, forming a complex by binding to chordin/short gastrulation (Sog) and BMP4 (Figure 1.3) (Chang *et al*., 2001). TWSG1 agonist BMP4, enhances the cleavage of chordin/sog by the proteolytic activity of the extracellular secreted xolloid (a member of the tolloid family of metalloproteases). TWSG1 competes with the residual fragments of chordin and binds directly with BMP4, activating its signaling pathway (Figure 1.3) (Oelgeschlager *et al*., 2000).

1.1.3 BMP and Hh interactions during embryonic development and diseases

During embryonic development, Hh and BMP signaling pathways are expressed in different locations of the tissue, which leads these two proteins to interact with each other in a cooperative or suppressive role. Among the three Hh ligands, Shh is expressed in the ventral side of the neural tube and remains vital in controlling ventral cell fate in neural vertebrate embryonic development (Liem *et al*., 1995) (Figure 1.1). Moreover, the essential role of Shh was demonstrated in knockout mice, where Shh leads to severe holoprosencephaly (Chiang *et al*., 1996). In addition, among the BMP ligands, BMP4 has been involved in the tissue patterning of several embryonic structures (Kishigami and Mishina, 2005). Research has demonstrated that BMP4 is expressed in the dorsal side of the neural

tube, controlling the patterning of the dorsal cell fate at the dorsal-ventral formation of the tube (Figure 1.1) (Liem *et al*., 1995).

During the post-embryonic intestinal development of amphibians, Shh induces the expression of BMP4 (Ishizuya-Oka *et al*., 2006). Moreover, in the hindgut development of embryonic mice, epithelium Shh and mesenchymal BMP4 are negatively expressed in mice with anorectal malformations (Sasaki *et al*., 2004). Interestingly, during normal anorectal development, these proteins interact cooperatively, and are positively and strongly expressed in the epithelium and mesenchyme of mice. Studies have shown that epithelial Shh can induce the expression of the mesenchymal BMP4, which leads to lamina and submucosa differentiation (Ishizuya-Oka *et al*., 2006). However, some research has demonstrated that Shh and BMP4 can suppress each other. During carcinogenesis, the upregulation of epithelial Shh induces the mesenchymal expression of BMP4. The activation and upregulation of the epithelial Hh molecules, including Shh, could be induced via bile and acid exposure (Wang *et al*., 2010). These conditions subsequently regulate the stromal expression of BMP4 in Barrett's metaplasia, activating the epithelial SRY (sex determining region Y)-box (SOX)-9, which reprograms the squamous epithelial cells of the esophagus to favor a columnar phenotype (Wang *et al*., 2010).

1.2 Liver anatomy

The liver is the largest internal organ in the adult human body, with a weight range of between 1.2 and 1.5 kg (Liaskou *et al*., 2012). In humans, the liver is

located in the upper right corner of the abdomen, to the right of the stomach and below the diaphragm. Moreover, the liver is unequally divided into two lobes: a larger right lobe and a smaller left lobe. In 1957, a surgeon, Claude Couinaud, divided the liver into 8 segments (Figure 1.4), and described the vascular and biliary branches in each segment. This subdivision assists surgeons performing liver resection, to ensure best strategies to reduce blood loss and for tumor removal in the case of liver carcinoma (Abdalla *et al*., 2002).

On a microscopic level, the liver is further divided into many lobules, which involve plates of hepatocytes with a hexagonal shape (Figure 1.5) (Ishibashi *et al*., 2009). Each liver lobule consists of a portal triad: hepatic artery, common bile duct and central hepatic vein, with hepatic sinusoids, hepatic macrophages (Kupffer cells), and the perisinusoidal space (space of Disse) located between the hepatocytes and the hepatic sinusoids (Figure 1.6). As the liver receives blood from two major blood vessels, the portal vein and hepatic artery, the blood accumulates into the hepatic sinusoids. The sinusoids contain a mixture of oxygenated and deoxygenated blood, which eventually drains into the inferior vena cava via the central vein. Furthermore, the liver consists of two major cell types: parenchymal and non-parenchymal cells. A hepatocyte is a parenchymal cell, which is the main cell of the liver, representing 70% of the human adult liver cells (Ishibashi *et al*., 2009). Non-parenchymal cells include bile duct cells (cholangiocytes), sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells (Ishibashi *et al*., 2009). The Kupffer cells, which line the wall of the hepatic

sinusoids, are macrophages of the monocyte macrophage system, which perform an important function in destroying bacteria, virus particles, damaged erythrocytes and other foreign materials that may damage the liver (Henson *et al*., 1966; Lehner *et al*., 2001; Willekens *et al*., 2005). In addition, the Kupffer cells can induce pro-inflammatory molecules, including interleukin-6 (IL-6) (Gao, 2012).

1.2.1 Embryonic liver development

Liver formation begins with the formation of the endoderm germ layer during the gastrulation (Zorn, 2008). The germ layer endoderm develops in response to the expression of TGF-β Nodal in a concentration-dependent manner (Shen, 2007). Subsequently, the endoderm develops a primitive gut tube that can be sub-divided according to its location: foregut, midgut and hindgut. Research in mice revealed that the organogenesis of the liver is initially derived from the ventral endodermal foregut at embryonic day (E.) 8.0 of the embryonic gestation period (Tremblay and Zaret, 2005). In the ventral foregut endoderm, the induction of the hepatic fate occurs at E.8.5 in response to patterning expression growth factors, including fibroblast growth factor (FGF) (Deutsch *et al*., 2001;LaBonne and Whitman, 1994), which is expressed from cardiac mesoderm development; and BMP4, which is expressed from the septum transversum mesenchyme (STM) (Rossi *et al*., 2001). At E.9.0, liver diverticulum begins, and the liver divides into two portions: anterior and posterior. The anterior section produces the liver and intrahepatic biliary tree, while the posterior part creates the extrahepatic biliary tree and the gallbladder (Roskams and Desmet, 2008). At E.9.5, the liver bud is formed when the hepatoblasts develop from the ventral foregut endodermal

epithelium and the endothelium as well as the STM. The proliferation and the survival of hepatoblasts occur in response to a number of extracellular molecules, including fibroblast growth factor (FGF) (Mavila *et al*., 2012), hepatocyte growth factor (HGF) (Schmidt *et al*., 1995), and wingless-related integration site (Wnt) signaling molecules (Tan *et al*., 2008). At E.10, the liver bud enters the growth process with assistance from the hematopoietic cells. Recent studies provided evidence that Hh ligands, including Shh (Hirose *et al*., 2009) and Patched (Ptch) (Sicklick *et al*., 2006), were highly expressed during E.11.5. Throughout the liver maturation process, from E.12.5 to 17.5, the expression of these ligands dramatically decreases to a complete lack of expression in the mature neonatal liver.

1.2.2 Differentiation of hepatocytes and cholangiocytes

The hepatoblasts fulfill a bi-potential role, as they are capable of creating both hepatocyte cells (primary liver cells) and cholangiocyte cells (bile duct cells) by E.13 (Rogler, 1997). Studies on embryonic liver mice found that several molecules, active during the differentiation of the hepatoblasts into hepatocytes, can be considered markers, such as hepatocyte nuclear factor 4 alpha (HNF-4α). The dramatic reduction of alpha-fetoprotein (AFP) and increased production of albumin during hepatocyte differentiation after 3-5 weeks of liver development, suggest these proteins may be markers of immature and mature cells, respectively (Cascio and Zaret, 1991; Freeman *et al*., 1981; Li *et al*., 2000). While the differentiation of the cholangiocyte cells entails a high expression of cytokeratin (CK)-19 and 7, TGF-β (Zorn, 2008) and SOX9 (Antoniou *et al*., 2009) that may

play an essential role in mediating the differentiation of the cholangiocytes. Specifically, the expression of SOX9 represents the earliest detectable marker of epithelial biliary cell differentiation, which begins at (E.11.5) of liver development and is located close to the portal vein, where the biliary tree remains (Antoniou *et al*., 2009). Moreover, at E.15.5-E.17, the biliary precursor cells that differentiated from the hepatoblasts undergo a series of tubulogenesis and cellular apoptosis mechanisms in order to maintain the shape of the bile duct; this process is known as ductal plate remodeling (Figure 1.7) (Lemaigre, 2003; Sergi *et al*., 2000). At E.15.5, the biliary cells begin to form a ring-like structure of mono-layered cells around the portal vein, forming the ductal plate, which subsequently develops a bi-layered structure E.16.5. At E.17, the bi-layered cells focally dilate the two cell layers and form the intrahepatic bile duct. Around birth, the cells are incorporated from the portal vein, where the remaining hepatoblasts surrounding the portal mesenchyme differentiate into hepatocytes (Lemaigre, 2003).

1.3 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is an aggressive primary tumor that arises from hepatocytes, which constitute the major cell type in the liver (Blonski *et al*., 2010; Taniguchi *et al*., 2002). Patients with HCC have a poor prognosis, with the five-year survival rate less than 5% (Clark *et al*., 2005). HCC is clinically silent until it becomes advanced or when the diameter of the tumor exceeds 10 cm (Motola-Kuba *et al*., 2006). More than 80% of HCC cases originate in a cirrhotic liver (Fan *et al*., 2009). Although HCC's etiology remains unknown, several studies have reported risk factors that may play a central role in its development.

1.3.1 Epidemiology

HCC is described as the fifth most common cancer worldwide, as it kills more than 500,000 people annually (Parkin *et al*., 2005). Specifically, this condition is the third and sixth most fatal malignant neoplasm among men and women respectively (Parkin *et al*., 2001). The incidence of HCC is strongly correlated with geographical location on the basis of exposure to HCC risk factors such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, and mycotoxin (aflatoxin-B1) (Sylla *et al*., 1999). However, the frequency of HCC differs among racial and ethnic groups within the same country.

The highest incidence of HCC cases caused by HBV and aflatoxin-B1 has been reported in Africa and Southern Asia (Yang and Roberts, 2010). In the United States of America (USA) and Europe, HCC is the ninth most fatal carcinoma among cancers, which results from the fact that HCV has been increasing during the past 40 years, with 90% of HCC cases positive for the HCV antigen (El-Serag, 2002). In Canada, HCC is reported to be the 19th most common malignant cancer in the Canadian population (Lin, 2009).

One epidemiological study reported that HCC incidence increased by 38% for both men and women from 1984 to 2000: from 3.98 per 100 000 to 5.5 per 100 000 in men, and from 1.6 per 100 000 2.2 per 100 000 in women (Dyer *et al*., 2005). However, the mortality rate was higher for men than women, 48% and 39%, respectively (Dyer *et al*., 2005).

1.3.2 Risk factors

HCC is a primary liver neoplasm with poor survival rates among patients of both sexes (Dyer *et al*., 2005). Although the etiological factors causing HCC remain poorly understood, several risk factors have been suggested to perform a central role during HCC carcinogenesis, including infection with viral hepatitis B (HBV) and hepatitis C (HCV); both are known to be positive in some HCC cases (Chang, 2007; El-Serag, 2002; Tanaka and Arii, 2009). Other common risk factors were also strongly correlated with HCC, including food, such as grain and corn, contaminated with aflatoxin-B1. This contamination results from poor quality food storage techniques, where the humidity and heat enhance fungus growth (Szymanska *et al*., 2009). Furthermore, excessive alcohol consumption represents another risk factor as it causes chronic cirrhosis of the liver, which may progress to a degenerative neoplasm and HCC (Yuen *et al*., 2002). Deficiency of a protease inhibitor, α-antitrypsin, is related to HCC carcinogenesis via liver cirrhosis (Kelly *et al*., 1979). A mutation of the Serpin Peptidase Inhibitor, Clade A (SERPINA1) gene, which encodes alpha-1 antitrypsin, leads to a deficiency of α-antitrypsin. The function of α-antitrypsin is to protect normal liver tissue from damage by powerful enzymes, such as neutrophil elastase secreted from white blood cells. Finally, hemochromatosis is a hereditary overload of iron that causes liver cirrhosis and later development into HCC (Stal *et al*., 1995).

1.3.3 Hepatocellular carcinoma pathogenesis

Risk factors create a favorable environment for chronic inflammation of the epithelial hepatocytes, which leads to liver cirrhosis and malignant

transformation. Specifically, chronic liver inflammation and liver cirrhosis have been strongly linked to the carcinogenesis of HCC (Blonski *et al*., 2010). Both of these conditions promote the major mechanisms of cancer, including cell proliferation, cell death (apoptosis) and cell migration (metastasis/invasion). Several types of molecular alterations have been strongly linked to promote these carcinogenesis mechanisms. Chronic inflammation and liver cirrhosis result in the expression of multiple chemokines and cytokines, including interleukin (IL)-8, IL-6, tumor-necrosis factor-α (TNF- α), and nuclear factor kappa B (NF-κB), which mediate cancer cell growth and survival (Cougot *et al*., 2007; Xiang *et al*., 2011). Moreover, dysregulation of tumor suppressors and oncogenes has been identified in HCC cases associated with chronic inflammation. The upregulation of IL6 via NF-κB leads to uncontrolled cell proliferation in HCC (Xiang *et al*., 2011). Transformed human hepatocyte cell lines (ChangX-34 and ChangX-31) that express HBx antigen, and HCC tissues infected with HBV have upregulated NF- κB, as well as enhanced cell proliferation and resistance to apoptosis (Um *et al*., 2007; Wang *et al*., 2004). In addition, studies have revealed that tumor suppressor 53 (TP53 or p53) gene is downregulated in HCC samples associated with chronic liver inflammation, leading to uncontrolled cell proliferation (He *et al*., 2010).

1.3.4 Clinical, laboratory diagnosis and tumor markers for HCC

Patients with HCC tumor usually show no signs of clinical symptoms. HCC is often diagnosed at the advanced stage and is sometimes mistaken for chronic

hepatitis and/or liver cirrhosis, which have symptoms of upper right quadrant abdominal pain, loss of weight, fever, anorexia, accumulation of abdominal fluid (ascites) and jaundice (yellowish pigment in the skin and the conjunctival membrane due to an increased bilirubin level in the blood) (Johnson, 1987).

Diagnostic imaging, including computerized axial tomography (CT) scan and/or magnetic resonance imaging (MRI), is used for an early detection of HCC tumor (Bolog *et al*., 2011). A CT scan is a combination of several X-ray images forming a cross-section or (slice) image of a specific area, to visualize an abnormal mass in the internal organs (Goldman, 2007). MRI uses a powerful magnetic field to send radio waves through the body to create a picture of the internal organs within the body, which is visualized on computer (Brody and Gooding, 1986).

Several laboratory tests are used to diagnose HCC. Many serum markers become elevated during chronic liver inflammation and remain continuously active during carcinogenesis; these markers include alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) (Gupta *et al*., 2003; Maeda *et al*., 1988). Specifically, AFP is a serum glycoprotein identified as a marker that helps to diagnose HCC (Kashyap *et al*., 2001). During liver development, the fetal liver expresses a high level of AFP, which subsequently decreases within 300 days after birth (Kashyap *et al*., 2001). Furthermore, AFP was detected in many patients with HBV, HCV and liver cirrhosis (Harada *et al*., 1980; Hiotis *et al*., 2012). CEA protein is normally expressed during embryogenesis in some organs,

including the liver (Huang *et al*., 1990). A high serum level of CEA expression was detected in number of HCC patients (Maeda *et al*., 1988).

Besides serum tests, tumor markers are widely used tissue tumor to diagnose HCC. Polyclonal-CEA (p-CEA) has a sensitivity of 80% in both well- to moderately-differentiated HCC tumors (Saad *et al*., 2004). Hepatocyte paraffin 1 (Hep Par 1) has a specificity and sensitivity of >90% in well- and moderately-differentiated HCC tumor (Fan *et al*., 2003). Glypican (GPC)-3 is a useful marker to diagnose 82-93% of moderately-differentiated HCC tumors, and 86-100% of poorly-differentiated HCC tumors (Yamauchi *et al*., 2005; Wang *et al*., 2006; Shafizadeh *et al*., 2008). These markers have several limitations. Specifically, p-CEA specificity is poor, because it may also be strongly expressed in other gastrointestinal tract (GIT) tumors, including hepatobiliary carcinomas (Lau *et al*., 2002). Hep Par 1 expression wanes to mild or negative in poorly-differentiated HCC tumors (Lugli *et al*., 2004). GPC3 is highly expressed in cirrhotic nodules of the liver, but negative to mild in most well-differentiated HCC tumors (Shafizadeh *et al*., 2008).

1.3.4.1 HCC tumor grading system

Tumor grading is a system used to identify the irregularity of the cells under the microscope, and to evaluate the severity of the tumor progression. Edmonson and Steiner first established the HCC grading system (Edmondson and Steiner, 1954). This system is based on the features of nuclei alone or the combination of nuclei features and micro-vascular invasiveness. The Edmonson and Steiner grading

system is composed of four grades: G1, well-differentiated tumor; G2, moderately-differentiated tumor, G3, poorly-differentiated tumor and G4, undifferentiated tumor, as defined in (Table 1.1).

1.3.5 Treatment by surgical resection

The treatment of choice for solitary HCC involves the partial surgical resection of the liver, known as partial hepatectomy (Kishi et al, 2011). Candidate patients for surgical resection should fulfill certain criteria which include: 1) normal liver function achieved by a biological assessment of the patients with cirrhosis according to the Child–Pugh classification (grade A and early grade B) (Table 1.2), 2) the number of lesions should not exceed 3, and 3) the tumor size should not exceed 5 cm in diameter (Duffy *et al*., 2008). However, a high concentration of bilirubin and the presence of portal hypertension can be used as criteria for HCC resection (Crocetti and Lencioni, 2008). A low bilirubin concentration and the absence of clinically significant portal hypertension improved the five-year survival rate by approximately 70% after surgical resection (Crocetti and Lencioni, 2008). However, this number was decreased to 50% in patients with portal hypertension and to 25% in patients with high levels of bilirubin and portal hypertension (Crocetti and Lencioni, 2008). Despite the successful rate of surgical resection, many reports have revealed its association with a high risk of cancer recurrence by more than 50% of patients (Shah *et al*., 2007).

1.3.5.1 Treatment by liver transplantation

Liver transplantation represents a more effective therapeutic option for HCC patients, as it eradicates both detectable and undetectable lesions. HCC patients that are candidates for liver transplantation must achieve the Milan criteria: the solitary HCC tumor size must not be greater than 5 cm in diameter, the total number of lesions must not exceed three, and there must be no sign of vascular invasion or extrahepatic spread (Tanwar *et al*., 2009). Research has shown that carefully selected patients undergoing liver transplantation reported an increase in the 3-month, 1 and 3-year survival rates 89%, 89%, and 81% respectively (Qasim *et al*., 2007). Moreover, many studies report disease recurrence is significantly lower in patients with a transplanted liver, with a recurrence rate below 15% compared to patients treated by surgical resection (Llovet *et al*., 2005).

1.4 Cholangiocellular carcinoma

Cholangiocellular carcinoma (CCA) is a malignant neoplasm originating from the bile duct (Khan *et al*., 2005). This condition represents the second most common primary malignant tumor of the liver, where it occurs in the extrahepatic and/or intrahepatic biliary tree (Tyson and El-Serag, 2011). CCA is clinically silent and difficult to diagnose until the advanced stages, thus explaining its status as the silent killer (Khan *et al*., 2005; Olnes and Erlich, 2004).

According to the World Health Organization (WHO), CCA is classified into two types: extrahepatic and intrahepatic (Hamilton and Aaltonen, 2000). Subsequently, extrahepatic CCA is further categorized into two types based on the

tumor's location in relation to the liver hilum: perihilar or Klatskin, and distal extrahepatic (Figure 1.8) (Charbel and Al-Kawas, 2011; Nakeeb *et al*., 1996; Reinhardt *et al*., 2005).

1.4.1 Epidemiology

CCA has been described as the second most common primary hepatic cancer, which is most commonly occurs in patients between 50 to 60 years old (Blechacz and Gores, 2008; Singal et al, 2011). Studies report a slight (3.4%) gender difference, suggesting that men are more susceptible to developing CCA than are women (Shaib *et al*., 2005; Shin *et al*., 2010). However, Welzel *et al*. debated this finding, reporting no significant gender differences in the occurrence of CCA (Welzel *et al*., 2007). In all probability, the association of CCA with gender may vary according to geographical location (Chung *et al*., 2009). The highest incidence of CCA occurs within Southeast Asia, and this cancer has been officially recognized as one of the major health issues in this region (Srivatanakul *et al*., 2010). The relatively high incidence of CCA in South and East Asia was strongly associated with the liver fluke infection, including the carcinogenic parasites *Opisthorchis viverrini* and *Clonorchis sinensis* (Mairiang *et al*., 2012; Pinlaor *et al*., 2004). Although the lowest incidence of CCA was identified in western countries (Shin *et al*., 2010), CCA has dramatically increased in western countries between 1975 and 2000 (Hammill and Wong, 2008). This increasing incidence has been strongly linked to the growing number of Asians in North America (Yossepowitch *et al*., 2004). Specifically, the two most important risk

factors, *O. viverrini* and *C. sinensis,* have been found among Asian immigrants and tourists in North America (Yossepowitch *et al*., 2004).

1.4.2 Risk factors

To date, there is no evidence of the main causes of CCA, however, studies and case reports suggested several risk factors play a role in CCA carcinogenesis. WHO has officially recognized that the consumption of contaminated raw fish represents a strong risk factor associated with CCA, especially fish infected by the liver flukes, *O. viverrini* and *C. sinensis,* which cause chronic inflammation of the bile duct system (Mairiang *et al*., 2012; Pinlaor *et al*., 2004). Hepatolithiasis, an accumulation of pigmented gallstones in the biliary tract tree with unclear causes, is strongly linked to parasitic infection (Shoda *et al*., 2003; Pilankar *et al*., 2003). Hepatolithiasis has a 10% incidence rate in Asia (Lesurtel *et al*., 2002). One genetic risk factor for CCA is primary sclerosing cholangitis (PSC), a chronic inflammation of the intra- and extrahepatic bile duct causing bile duct obstruction and bile duct cirrhosis. The incidence of CCA in PSC is between 6% and 30% (Lee and Practice Guideline Committee of the American College of Gastroenterology, 2002). Chemicals such as nitrosamines, used in pesticides, rubber products and cigarettes are carcinogenic, specifically associated with hepatobiliary carcinogenesis (Preussmann, 1984). Chemicals used in radiation exposure, such as Thorotrast, are also associated with the development of CCA. Thorotrast, a 25% colloid solution of dextrin and thorium dioxide (ThO2), was mostly used in Germany and in the USA in 1930's and 1940's as a versatile medical radiography contrast agent (Ohshima *et al*., 1994; Charles *et al*., 2003;

Lee *et al*., 1996). Other risk factors are weakly associated with CCA carcinogenesis, such as HBV and HCV (Lee *et al*., 2008), oral contraceptives (Kuper *et al*., 2001), and cigarette smoking (Aljiffry *et al*., 2009).

1.4.3 Cholangiocellular carcinoma pathogenesis

The previously mentioned risk factors create a favorable environment for chronic inflammation of the epithelial bile duct cells, leading to malignant transformation. Etiological and experimental evidence indicates that cholangitis (chronic inflammation of the biliary tree), and cholestasis (obstruction of the biliary tree disturbing the bile flow), are the main causes of the development of CCA (Zecca *et al*., 2006). Both conditions (chronic inflammation, obstruction) promote the major mechanisms of all cancers, including cell proliferation, cell death (apoptosis) and cell migration (metastasis/invasion) via DNA damage, oncogenes and tumor suppressor genes (Blechacz and Gores, 2008). Several types of molecular alterations are strongly linked to the promotion of these carcinogenesis pathways. During chronic inflammation of the bile duct, cholangiocytes and inflammatory cells express multiple chemokines and cytokines, including IL-6, a proinflammatory cytokine, is associated with CCA carcinogenesis (Isomoto, 2009; Wehbe *et al*., 2006). Specifically, bile acids and IL-6 control bile duct cell growth and cell survival during chronic inflammation by regulating the overexpression of the potent anti-apoptotic B-cell lymphoma (Bcl)-2 protein and induced myeloid leukemia cell differentiation protein (Mcl)-1 (Kobayashi *et al*., 2005). An abnormal expression of the K-ras oncogene was reported in 100% of CCA cases, and tumor suppressor gene (TP53) in up to 37% of CCA cases,

indicating that the alteration of these genes is highly associated with the progressive phenotype of this cancer (Isa *et al*., 2002). Moreover, investigations have revealed that *O. viverrini* can co-contribute to CCA oncogenesis because of the host-parasite interaction. Deregulation of platelet-derived growth factor alpha (PDGFA) by *O. viverrini* infection promotes carcinogenesis by downregulating many anti-proliferative and angiogenesis pathways (Boonjaraspinyo *et al*., 2012).

1.4.4 Clinical, laboratory diagnosis and tumor markers for CCA

Patients with CCA, are usually diagnosed with the advanced stage of the disease, and complain of the same symptoms as for HCC including abdominal pain, fever, anorexia and jaundice (Al-Bahrani et al, 2013). Imaging tools used for diagnosis include: CT scan and MRI are usually performed on patients that are suspected to have CCA tumor.

In cholangitis and cholestasis, the serum level of bilirubin, alkaline phosphatase, and gamma-glutamyltransferase are usually measured and elevated in CCA cases (Andraus *et al*., 2011; Castro-e-Silva *et al*., 1990; Olnes and Erlich, 2004). Other serum markers are elevated in CCA cases, including carbohydrate antigen 19-9 (CA 19-9) in 85% of CCA cases, CA-125 in 40%-50% of CCA cases, and CEA in 30% of CCA cases (Khan *et al*., 2002; Vauthey and Blumgart, 1994). However, these markers have certain limitations in the diagnosis of CCA; for instance, CA-125 and CEA are significantly elevated in other GIT carcinomas (Kochi *et al*., 2000; Omar *et al*., 1989). Also, the serum level of CA 19-9 depends upon the Lewis (Le) phenotype. The Lewis blood group is composed of two main antigens

(Le^{a} and Le^{b}), which are carbohydrate structures that form epitopes on the glycoproteins (Vestergaard *et al*., 1999). CA 19-9 was reported as undetectable in approximately 10% of populations with negative Lewis phenotypes (Vestergaard *et al*., 1999).

Tumor markers are also widely used in immunohistochemistry methods to diagnose CCA in human liver tissues: p-CEA in 100% of CCA cases (Lau *et al*., 2002), monoclonal antibody (MOC)-31, also known as epithelial specific antigen/Ep-CAM (epithelial cell adhesion molecule) in 93% of CCA cases (Morrison *et al*., 2002), CK7 in 97%-100% of CCA cases, and CK19 in 77%-80% of CCA cases (Stroescu *et al*., 2006; Vestergaard *et al*., 1999). However, these markers possess limitations, such as a lack of specificity. For example, p-CEA is also detected in HCC and other GIT carcinomas. Moreover, cytokeratin 7 (CK7) is expressed in both malignant and non-malignant liver bile ducts (Stroescu *et al*., 2006; Yabushita *et al*., 2001).

1.4.4.1 CCA tumor grading system

The tumor grading system is classified into 4 grades (G1-G4) according to the progression of the CCA tumor, and is based on percentage of the gland formation within the tumor (Bosman *et al*., 2010) (Table 1.3). G1 is a well-differentiated tumor where CCA tumor cells resemble normal bile duct cells; composed entirely of glands or less than 5% solid or cordlike growth patterns (change in bile duct shape from circular to narrow elongated form resembling a cord). G2 is a moderately-differentiated tumor where the tumor cells have more than 5% but less

than 50% solid or cordlike growth patterns. G3 is a poorly-differentiated tumor where the tumor is composed of 50% to 100% solid or cordlike growth patterns. G4 is an undifferentiated tumor where the tumor is composed of less than 5% glands (Bosman *et al*., 2010).

1.4.5 Treatment by surgical resection

Surgical liver resection, also known as a partial hepatectomy, represents the preferred treatment choice for patients with extrahepatic bile duct CCA (Burke *et al*., 1998). In fact, the only curative treatment for patients with CCA involves complete surgical resection the CCA tumor with a histological negative margin (tumor free at the tissue edges after resection) (Burke *et al*., 1998). Several criteria must be considered for surgical resection of the bile duct tumor, including the size of the tumor, the location of the tumor in relation to the biliary tree, the extent of vascular invasion, the atrophy of the hepatic lobe, and the involvement of the main portal vein, as well as the presence of liver, cardiovascular and metastatic diseases (Blechacz and Gores, 2008; Denys *et al*., 2002). Additionally, other factors, such as the patient's nutritional status, radiological data, magnetic resonance cholangiopancreatography and clinical laboratory tests (serum albumin and bilirubin levels) are predictors for liver failure and death after surgery (Anderson *et al*., 2004). The five-year survival rate for patients undergoing surgical resection of the bile duct tumor is generally between 8% and 44% (Anderson *et al*., 2004). While patients undergoing negative margin surgery experienced a five-year survival rate outcome of 19%-47% (Anderson *et al*., 2004).

1.4.5.1 Treatment by liver transplantation

Another therapeutic option for CCA patients, liver transplantation, remains controversial. This treatment is not recommended due to the high CCA recurrence rate. Specifically, Meyer *et al*. reported that overall, 51% of 207 patients experienced recurrence, and 84% of recurrence happened within 2 years after liver transplantation, with an overall 5-year survival rate of 23% (Meyer *et al*., 2000). However, some studies showed an improvement in the survival rate using the Mayo Clinic protocol, which entails a combination of chemotherapy (administration of oral or intravenous cytotoxicity drug with cellular killing property), external beam radiotherapy (a large amount of radiation externally pointed at the tumor location) and brachytherapy (internal radiation therapy uses to deliver radiation directly to the tumor) following liver transplantation in patients with extrahepatic CCA (Rosen *et al*., 2008). Patients that are eligible for the Mayo Clinic protocol must exhibit an early stage of CCA that arises in the setting of underlying PSC and is deemed unresectable (Rosen *et al*., 2008). Candidacy for this protocol also depends upon the size of the tumor, which should be limited to 3 cm with no evidence of intrahepatic or extrahepatic metastases, and the patient must have no history of tumors within the past five years. Most importantly, patients with intrahepatic CCA and gallbladder tumors are excluded from this protocol. In extrahepatic CCA patients, the outcome of the Mayo Clinic protocol showed a 71% increase in the five-year survival rate (Rosen *et al*., 2008).

1.5 Cell line models for HCC and CCA

Malignant cell lines are immortalized primary monolayer cells sub-cultured from a primary culture (Masters, 2000). Primary culture is a group of cells that are harvested directly from a tumor and grown in media with fetal bovine serum (FBS) in the presence of carbon dioxide (CO_2) and a humid environment (Masters, 2000). Cell lines are widely used in many studies to understand the pathological mechanism of human diseases such as cancer.

1.5.1 HCC cell lines

HepG2 and HuH-7 are both homogenous primary HCC cells derived from well-differentiated (G1) HCC tumors from a 15-year-old Caucasian American, and a 57-year-old Japanese male, respectively (Aden *et al*., 1979; Nakabayashi *et al*., 1982). (Table 1.4) Both cell lines are immortalized with an epithelial-like structure (Aden *et al*., 1979; Nakabayashi *et al*., 1982).

1.5.2 CCA cell lines

Human CCA cell lines, including OZ and HuH28 cell lines were derived from poorly-differentiated (G3) extra- and intrahepatic CCA tumors from a 71-year-old Japanese male, and a 37-year-old Japanese female, respectively (Homma *et al*., 1987; Kusaka *et al*., 1988) (Table 1.5). OZ and HuH28 cell lines histologically have an epithelial-like structure with and without gland formation (Homma *et al*., 1987; Kusaka *et al*., 1988).

HuCC-T1 is another CCA cell line was extracted from patient with an intrahepatic moderately-differentiated (G2) CCA tumor of a 56-yr-old Japanese male (Miyagiwa *et al*., 1989) (Table 1.5). Histologically, this cell line has an epithelial- and gland-like structure.

1.5.2.3 Normal liver cell line

THLE-3 is an imortalized normal hepatocyte cell line with an epithelial-like morphology, extracted from an adult "immediate autopsy" donor with a healthy liver (Pfeifer *et al*., 1993). Unlike HCC cell lines, the lifespan of normal hepatocytes is very short, so they were immortalized by transformation with simian virus 40 (SV40) large T antigen, which has proto-oncogen activity (Pfeifer *et al*., 1993).

1.6 Rationale of this study

Liver cancer is one of the most fatal cancers, as it kills over 695,000 people worldwide per year (Lee *et al*., 2010). Based on worldwide case reports, the percentage of HCC and CCA neoplasms vary according to geographical region, gender, and race (Altekruse *et al*., 2009; McLean and Patel, 2006). Moreover, liver cancers are among the most difficult cancers to identify because they are usually diagnosed at advanced stages, hence their reputation as silent killers (Motola-Kuba *et al*., 2006; Khan *et al*., 2005). While the etiological mechanism of liver cancers remains undetermined, several studies have suggested that liver cancer is strongly associated with environmental risk factors, such as hepatitis viral infection, liver fluke infestation, chemical and radiation exposure, and

chronic hepatobiliary inflammation. The only therapeutic option that has proven to work effectively involves surgical resection with patients who possess small, early-stage tumors.

Certain tumor markers and laboratory serum tests have been widely used to diagnose liver cancers. However, these markers have certain limitations in identifying HCC and CCA, due to a lack of sensitivity and/or specificty. For example, CA 19-9 lacks sensitivity and depends upon the Lewis phenotype; it was reported as undetectable in approximately 10% of populations with negative Lewis phenotypes (Vestergaard *et al*., 1999). An example for low specificity, CK7 is expressed in both malignant and non-malignant bile ducts (Stroescu *et al*., 2006; Yabushita *et al*., 2001). Furthermore, the similarity of both HCC and CCA tumors requires a marker that differentiates one condition over the other.

Morphogensis proteins have an essential role during embryogenesis (Hartenstein, 1989; Megason and McMahon, 2002), and may be reactivated in the process of cancer development and progression (Liao et al., 2009; Nhieu et al., 1999; Rich et al., 2001). Alterations in morphogen proteins such as BMP4 and Shh, which enhance cell proliferation, are associated with cancer development (Hahn et al., 1996; Guo et al., 2012; Lorente-Trigos et al., 2010).

The role of BMP4 in carcinogenesis is still controversial as to whether it is a promoter of tumor progression and invasion, or a tumor suppressor.

Overexpression of BMP4 induced colonic cancer cell migration (Deng et al., 2007). In gastric carcinoma cell lines obtained from patients with metastasis and poorly-differentiated tumors, BMP4 expression was strongly associated with tumor progression and cell migration (Katoh and Terada, 1996). However, a more recent study reported overexpression of BMP4 in gastric carcinoma cell lines established from poorly differentiated gastric tumor induced cell cycle arrest via p21, therefore acting as a tumor suppressor (Shirai et al., 2011). The colon and stomach have large luminal surface areas, and tumors in these organs have a high expression of BMP4. The bile duct also has a large luminal surface area. Is it possible that the tumors of the bile duct also have a high expression of BMP4 that may be greater than in hepatocellular tumors?

In pancreatic and gastric tumors, Shh is reported to be strongly associated with tumor progression; expression is greater in G1 (well-differentiated) than G3 (poorly-differentiated) tumors (Jang et al., 2007; Ma et al., 2005).

Taken together, the expression of BMP4 and Shh in GIT carcinomas is associated with the tumor progression and tumor metastasis via EMT, suggesting that BMP4 and Shh may have essential roles in polarizing and remodeling the cellular morphology (cytoskeleton). Studies have reported BMP4 and Shh interact with each other during embryonic development in a concentration-dependent manner, and also during carcinogenesis, promoting tumor progression and invasiveness (Liem *et al*., 1995; Ishizuya-Oka *et al*., 2006; Wang *et al*., 2010). Moreover,

TWSG1 can act as an inhibitor or promoter for BMP4 (Chang *et al*., 2001; Oelgeschlager *et al*., 2000). Mutation or loss of TWSG1 may lead to embryonic developmental defects and abnormalities (Petryk *et al*., 2004). To the best of my knowledge the role of TWSG1 in liver cancer has not been investigated previously. The usefulness of BMP4, Shh and TWSG1 in differeniating HCC and CCA epithelial tumors is unknown.

1.6.1 Hypotheses

1-Differential expression of bone morphogenetic protein 4 (BMP4), sonic hedgehog (Shh) and twisted gastrulation protein 1 (TWSG1) proteins distinguish cholangiocellular carcinoma (CCA) from hepatocellular carcinoma (HCC).

2-The expression of BMP4, Shh and TWSG1 proteins is increased with decreased differentiation of CCA and HCC tumors.

1.6.2 Objectives

The following objectives providing a systematic approach to test this hypothesis:

1-To investigate the differential expression patterns of TWSG1, BMP4 and Shh in formalin fixed paraffin embedded human liver tumors (cholangiocellular carcinoma and hepatocellular carcinoma) using immunohistochemistry.

a) To investigate the association of BMP4, TWSG1 and Shh protein expression with CCA and HCC tumor grade.
b) To investigate the co-localization of BMP4 and TWSG1, and Shh and BMP4 in human CCA and HCC tumors.

2-To investigate the pattern expression of BMP4, TWSG1 and Shh proteins in cell lines derived from well- to poorly-differentiated human CCA and HCC tumors using the Western blot method.

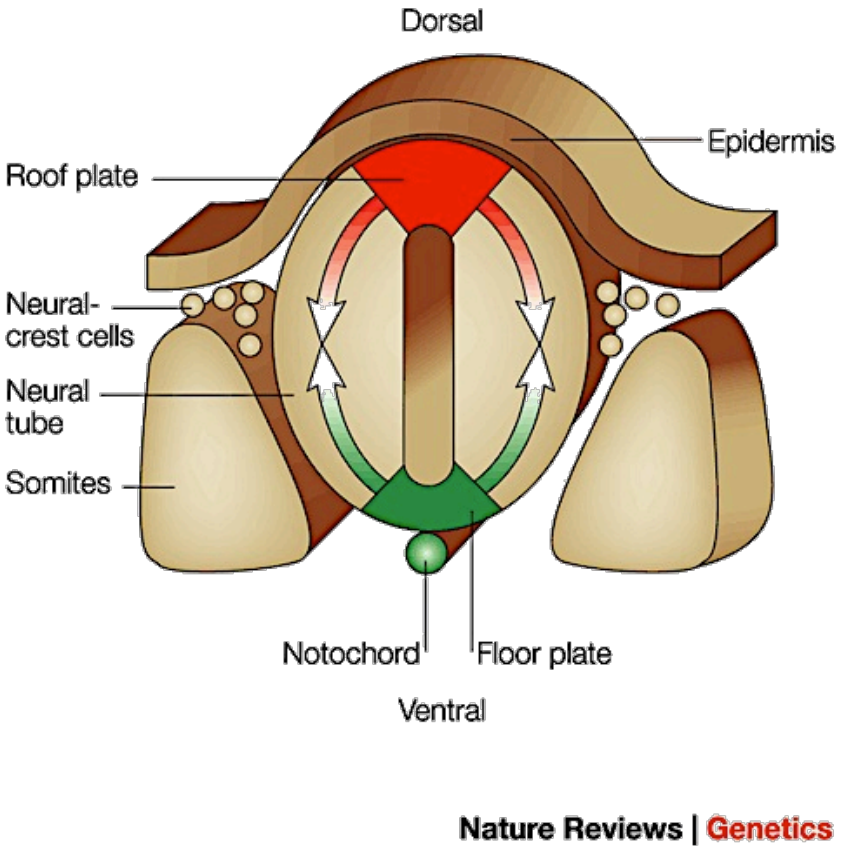

Figure 1.1. Shh and BMP4 role in neural tube formation. The gradient expression of sonic hedgehog (Shh) (green) by the notochord and the floor-plate cells at the ventral midline patterns. The gradient expressions of bone morphogenetic protein (BMP)-4 (red), which is expressed by the epidermis and the roof-plate cells at the dorsal midline, pattern the dorsal neural tube. (Tabata, 2001)

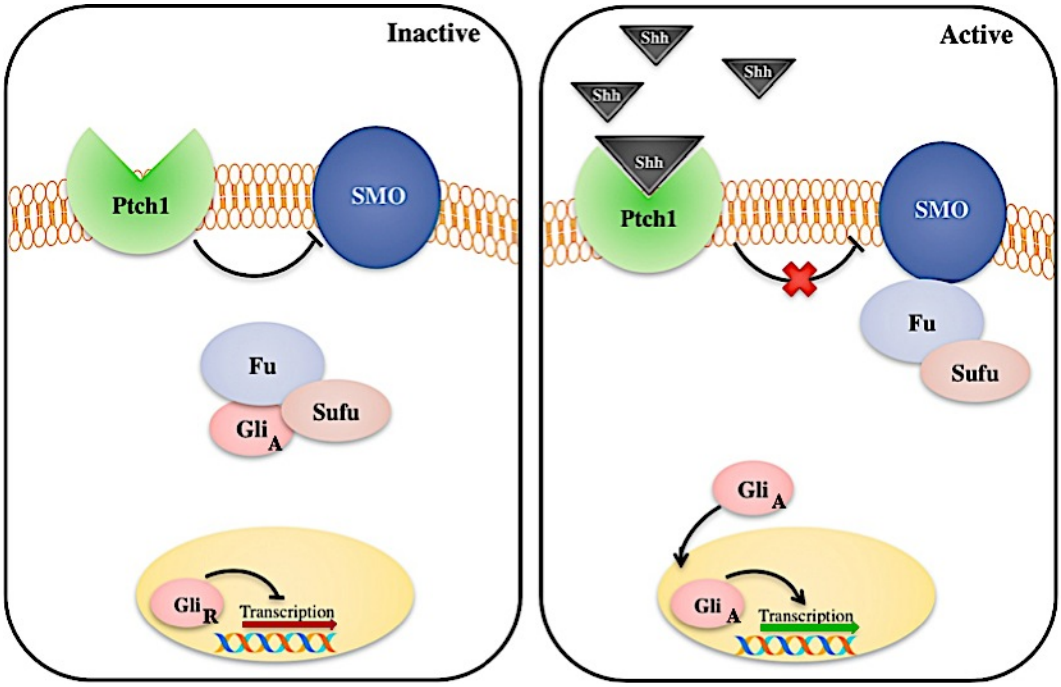

Figure 1.2. Sonic hedgehog (Shh) signaling transduction pathway. In the absence of Shh ligand (left panel), Patched (Ptch)-1 represses the activity of signaling receptor smoothened (SMO). Fussed (Fu) and suppressor of fused (Sufu) form an inhibitory complex with Gli_A transcription factor activator, cleaving it to prevent it from entering the nucleus. Nuclear Gli_R is processed to form a transcriptional repressor (green). In the presence of Shh ligand binding to its receptor Ptch-1 (right panel), SMO is no longer inhibited, and releases the inhibitory complex of Fu and Sufu, which turns off Gli_A cleavage processing. Gli_A translocates to and enters the nucleus where it induces the expression of target genes (red) (Dessaud *et al*., 2008).

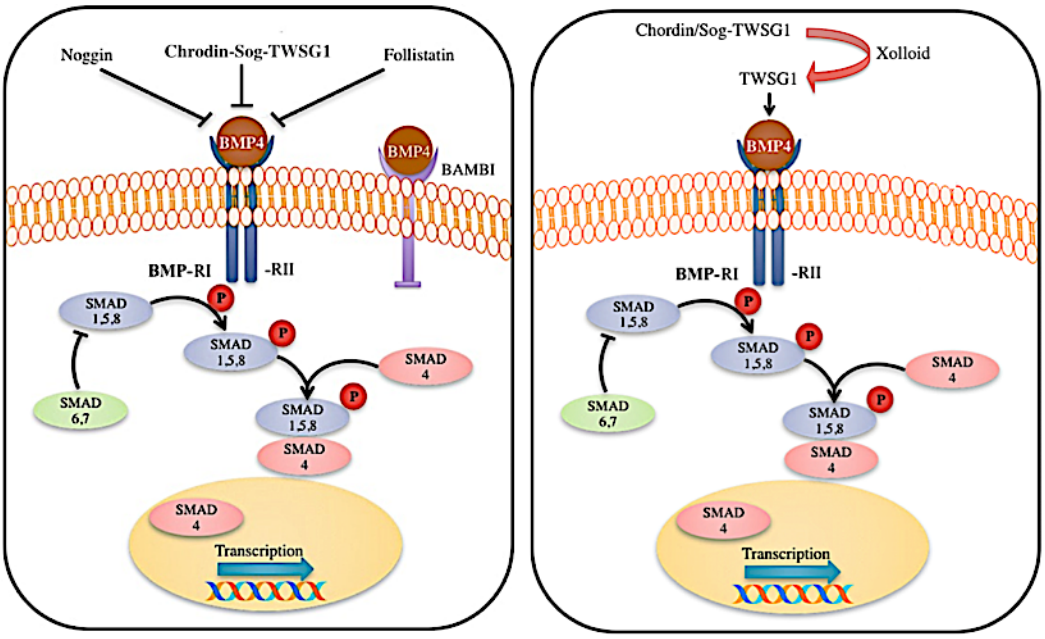

Figure 1.3. Bone morphogenetic proteins 4 (BMP4) signaling pathway: The canonical BMP signaling pathway is initiated by the binding of BMP to heterodimers of BMP4 receptors (BMP-RI) and (BMPRII). This leads to activation of Smad1/5/8, which forms a heteromeric complex with Smad4 prior to translocation to the nucleus. In the left panel illustrated the extracellular inhibitors of BMP4 such as noggin, chordin-Sog-TWSG1, follistatin and BAMBI, and the intracellular inhibitory Smads such as Smad7 provide negative feedback regulatory mechanisms. In the right panel TWSG1 binds directly to BMP4 after the cleaved of chrodin/sog-TWSG1 by xolloid (tolloid metalloproteases). (Chen *et al*., 2004; Walsh *et al*., 2010)

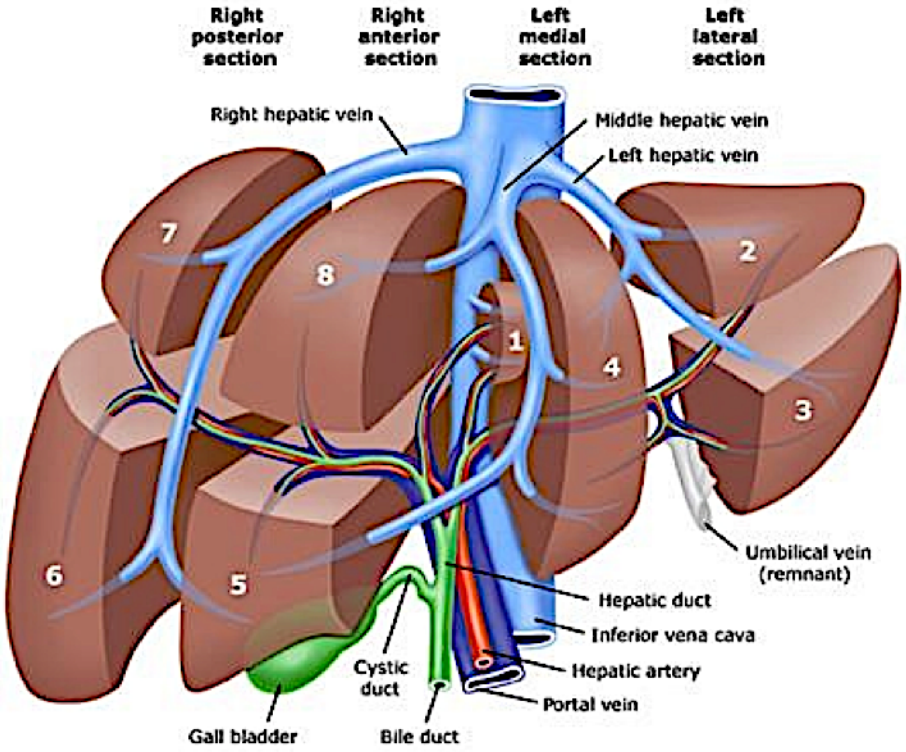

Figure 1.4. Liver anatomy. The classification of the right and the left lobes of the human liver is labelled. Each lobe is further divided into 4 segments (Schiff *et al*., 2007).

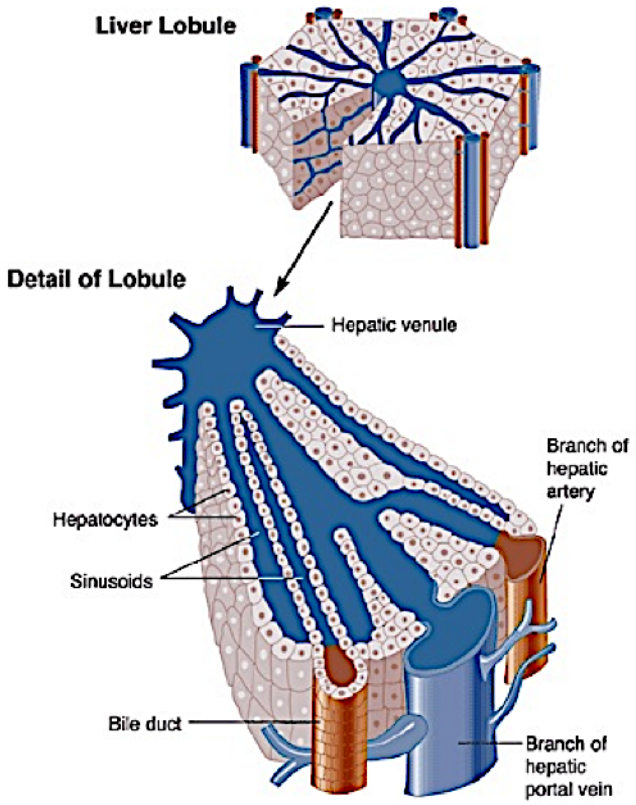

Figure 1.5. Liver lobule. Liver lobules consist of plates of hepatocytes with a hexagonal shape. Each lobule consists of a portal triad: hepatic artery, common bile duct and central hepatic vein with hepatic sinusoids (Cunningham and Van Horn, 2003).

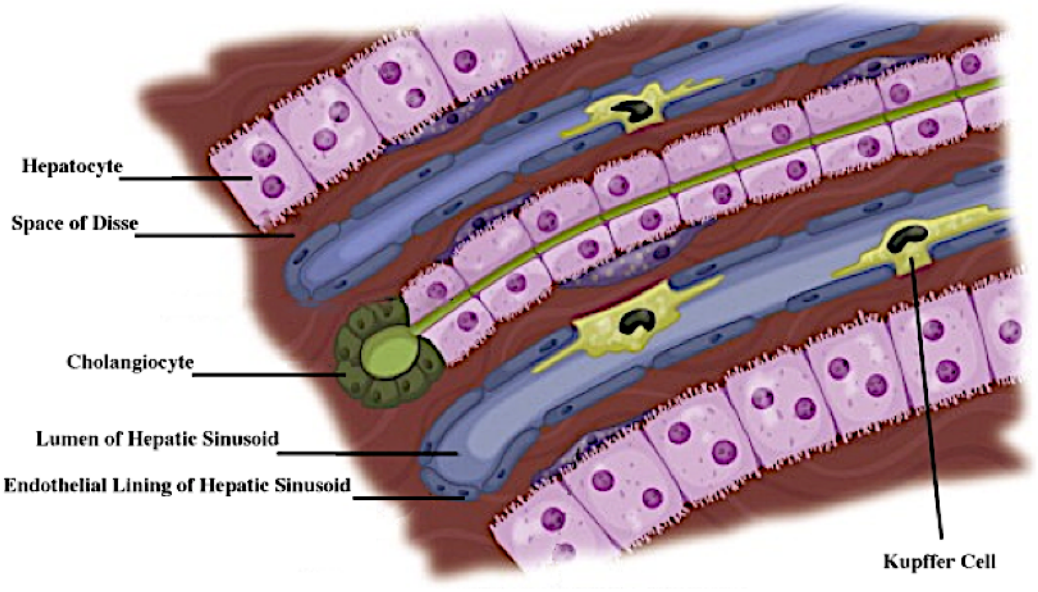

Figure 1.6. Liver cells. Diagram illustrates different cell types in the liver. Parenchymal hepatocytes are arranged in single-cell thick plates. Non-parenchymal cholangiocytes line the bile duct. Bile canaliculi run along the surface of hepatocytes. Hepatic sinusoids are small blood vessels that contain a mixture of oxygenated and deoxygenated blood lined endothelial and Kupffer cells, which line the wall of the hepatic sinusoids, are macrophages of the monocyte macrophage system. The perisinusoidal space (space of Disse) located between the hepatocytes and the hepatic sinusoids. (Baxter *et al*., 2010)

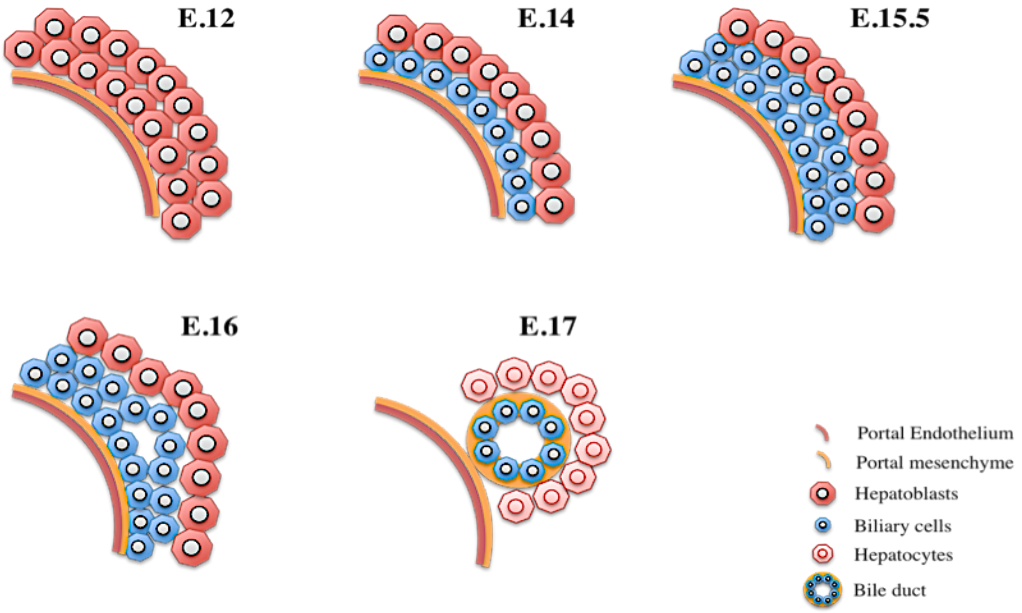

Figure 1.7. Bile duct formation. At E.12 the hepatoblasts starts to differentiate into biliary cells. At E.15.5 the biliary cells begin to form mono-layered cells around the portal mesenchyme known as the ductal plate, which then becomes bi-layered around E.16. At E.17 the bi-layered cells focally dilate the ductal plate and form the intrahepatic bile duct, which are then incorporated from the portal mesenchyme around birth, where the remaining hepatoblasts around the portal mesenchyme differentiate into hepatocytes. (Lemaigre, 2003; Sergi *et al*., 2000)

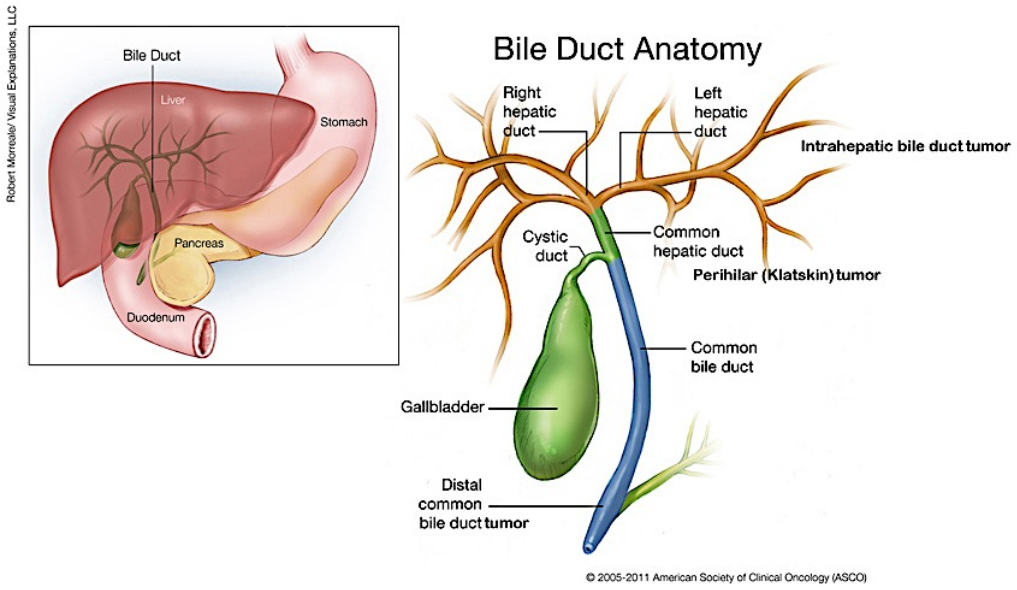

Figure 1.8. Bile duct tumor classification. (Brown) represents Intrahepatic CCA; (green) represents perihilar (klastkin); and (Blue) represents distal extrahepatic CCA tumor. (American Society of Clinical Oncology (ASCO)-Cancer.Net)

Table 1.1. Hepatocellular carcinoma grading system. The of Edmondson and Steiner grading system for hepatocellular carcinoma focuses on the architecture and the characteristics of the tumor cell, nucleus, cytoplasm and the presence of the liver acini (Edmondson and Steiner, 1954).

Tumor grade	Nuclear characteristics	Cytoplasmic characteristics	Acini	Note
G1	The well differentiated tumor G1 is very rare and mostly resemble to G2			
G2	Larger and hyperchromatic than grade 1	Abundant and acidophilic	Frequently seen and are variable in size. Also filled with bile or protein precipitate	Cell borders are sharp and clear cut
G3	Larger and hyperchromatic than grad 2. Also occupy a greater proportion of the cells	Granular and acidophilic but less than grade 2	Less often, and filled with bile or protein precipitate	More single cells were seen in the intravascular growths
G4	Larger and intensely hyperchromatic than grad 3. Also occupy a high percentage of the cells	Variable in amount, scanty and has fewer granules than grade 3	Rarely seen	Growth pattern is medullary in character, trabeculae difficult to find, and cell masses seem to lie loosely without cohesion in vascular channels

G1, well-differentiated tumor; G2, moderately-differentiated tumor; G3, poorly-differentiated tumor; G4, undifferentiated tumor

Table 1.2. Child–Pugh classification. Scoring system applied to candidate patients with liver cirrhosis for liver resection, based on laboratory and clinical parameters (Duffy *et al*., 2008).

Lab Parameters	**Measures**			**Interpretation**
	Point 1	Point 2	Point 3	
Bilirubin (mg/dL)	< 2	2 - 3	> 3	Grade A 5-6 Points Grade B 7-9 Points Grade C 10-15 Points
Albumin (g/dL)	> 3.5	2.8 - 3.5	< 2.8	
Prothrombin time (Seconds)	1 - 3	4 - 6	> 6	
International normalized ratio	< 1.7	1.7 - 2.3	> 2.3	
Clinical Parameters				
Hepatic encephalopathy (grade)	None	I – II	III - IV	
Ascites	None	Mild - Moderate	sever	

Table 1.3. Cholangiocellular carcinoma grading system. The grading system of cholangiocellular carcinoma "adenocarcinoma" is based on the gland formation within the tumor (Bosman *et al*., 2010).

Tumor grades	Interpretation
G1	The tumor is composed entirely of glands or has less than 5% solid or cordlike growth patterns
G2	The tumor has more than 5% but less than 50% solid or cordlike growth patterns
G3	The tumor is 50% to 100% solid or cordlike growth patterns
G4	The tumor has less than 5% of glands

G1, well-differentiated tumor; G2, moderately-differentiated tumor; G3, poorly-differentiated tumor; G4, undifferentiated tumor

Table 1.4. Parameters of hepatocellular carcinoma (HCC) cell lines (Aden *et al*., 1979; Nakabayashi *et al*., 1982)

Cell lines	HepG2	HUH7
Species	Human	Human
Gender	Male	Male
Age/Year	15	57
Tumor type	HCC	HCC
Tumor grade	Well-differentiated (G1)	Well-differentiated (G1)
Metastatic	No	No
Tumor source	-	-
Doubling time	48h	35.8h

Table 1.5. Parameters of cholangiocellular carcinoma (CCA) cell lines (Homma *et al*., 1987; Kusaka *et al*., 1988; Miyagiwa *et al*., 1989)

Cell lines	**OZ**	**HuH28**	**HuCCT1**
Species	Human	Human	Human
Gender	Male	Female	Male
Age/Year	71	37	56
Tumor type	Extrahepatic CCA	Extrahepatic CCA	Intratrahepatic CCA
Tumor grade	Poorly-differentiated (G3)	Poorly-differentiated (G3)	Moderately-differentiated (G2)
Metastatic	Yes	-	-
Tumor source	Intrahepatic	Intrahepatic	Intrahepatic
Doubling time	48h	80h	74h

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Chapter 2

Twisted gastrulation expression in cholangiocellular and hepatocellular carcinoma

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2.1 Introduction

Transforming growth factor β (TGF-β) superfamily members regulate a number of different developmental processes and disruption of their signaling has been implicated in a variety of diseases including cancer (Guo and Wang, 2009). The TGF-β superfamily comprises a large and diverse group of polypeptide morphogens including the prototype of the family with the isoforms of TGF-β, bone morphogenetic proteins (BMPs), growth and differentiation factors, activins (A and B), inhibins (A and B), nodal, leftys (1 and 2) and Muellerian inhibiting substance (Knight and Glister, 2006). The TGF-β superfamily is known to be involved in embryonic development, adult tissue homeostasis, tissue repair and pathogenesis of oncological diseases. Dysregulation of BMPs has been implicated in epithelial and mesenchymal tumorigenesis, including gastrointestinal and genitourinary tumors of mice and humans (Gordon and Blobe, 2008; Guo and Wang, 2009; Simank *et al*., 2001). The diverse nature and concentration-dependent signaling of BMPs requires that their function be tightly regulated and extracellular BMP-binding proteins constitute the first level of regulation of BMPs. BMP4 is a critical signaling molecule required for the early development of the embryo and differentiation of ectodermal tissue (Passa *et al*., 2011).

Twisted gastrulation (TWSG1) is an example of an extracellular secreted protein, which has been shown either to inhibit or promote BMP activity (Larrain *et al*., 2001; Petryk *et al*., 2005; Ross *et al*., 2001). Mice that are deficient in TWSG1 have a variety of developmental and postnatal defects such as craniofacial and

forebrain defects (Billington *et al*., 2011; Petryk *et al*., 2004; Sun *et al*., 2010). However, little is known about the role of TWSG1 in human pathology.

Cholangiocellular carcinoma (CCA) and hepatocellular carcinoma (HCC) are the most common types of liver cancer with poor prognosis worldwide. The highest incidence of CCA cases was reported in Southeast Asia associated with liver fluke infestation such as *Opisthorchis viverrini* and *Clonorchis Sinensis* (Mairiang *et al*., 2012; Pinlaor *et al*., 2004). The highest incidence of HCC cases is associated with HBV and aflatoxin-B1 and has been reported in Africa and Southern Asia (Yang and Roberts, 2010).

The expression of TWSG1 in human malignant epithelial tumors of the liver, CCA and HCC, has not been previously investigated (See section 1.6). Developmental processes of ontogenesis and phylogenesis are frequently reactivated during oncogenesis. The aim of this study is investigate the differential expression of BMP4 and TWSG1 proteins in CCA and HCC with tumor grade.

2.2 Materials and methods

2.2.1 Tissue samples and microarray

This study was approved by the institutional Human Research Ethics Board (University of Alberta, Edmonton, Canada; HREB approval #20274) and operationally by the provincial health care provider (Alberta Health Services). A retrospective search from July 2008 through December 2010 was carried out to

obtain tumor specimens of hepatocellular carcinoma (HCC) and intrahepatic cholangiocellular carcinoma (CCA) from the University of Alberta hospital archive. Qualifying reports were individually reviewed and tumors >1 cm were included in the study. Archived slides stained with hematoxylin and eosin (H&E) (Figure 2.1) and were reviewed by two pathologists (CS and BC) to assess tissue viability and consistency of diagnosis including cells morphology, cells necrosis, cells death and mitotic cells activity according to the College of American Pathologists (CAP) (Linda *et al*., 2011; Arief *et al*., 2011). Tissue microarrays (TMAs) were made using the Beecher tissue microarrayer (model TMA-1, Estigen, Estonia). Briefly, after selecting the sample site on the corresponding H&E slides, the marked sites located on the donor block. The tissue microarrayer is used for extracting 1.5 mm diameter cylindrical tissue cores from different donor blocks and re-embedding into a single microarray (recipient) block (Kononen *et al*., 1998).

2.2.2 Hematoxylin and eosin stain

A Discovery XT Ventana autostainer (Ventana Medical Systems, Inc, Tucson, Arizona, USA) was used in the Division of Anatomical Pathology in the Department of Laboratory Medicine and Pathology at the University of Alberta hospital for the H&E stain procedure. TMA samples were cut at 5-6 μm, deparaffinized in xylene (4X 1 min each) and rehydrated through a series of graded alcohols (ethanol 3X 100% 1 min each, 1X 95% and 1X 70% 1 min each). The sections were then rehydrated in tap water for 1 min. Sections were stained 4 min with hematoxylin (Leica Surgipath Canada product Ins, Concord, ON,

Canada). Sections were washed with tap water and then incubated with 10% aqueous differentiating reagent (define MX-aq) for 1 min (Leica Surgipath Canada product Ins, Concord, ON, Canada), and washed with tap water for 1 min. The slides were then incubated with 6% blue buffer 8 (lithium carbonate) for 1 min (Leica Surgipath Canada product Ins, Concord, ON, Canada) and washed with tap water for 1 min. The slides were then stained with eosin for 1 min, dehydrated (3X 1 min each) with 100% ethanol and cleared (3X 1 min each) with xylene and mounted with non-aqueous mounting media (paraffin oil) and covered with a coverslip.

2.2.3 Immunohistochemistry and scoring

Immunohistochemistry and quality controls were performed by Jolene Johnston to examine the presence and localization of TWSG1 in the tumor tissue as previously described (Sun *et al*., 2010). The 5 μm tissue sections were dewaxed and rehydrated, and then stained using a Discovery XT Ventana Autostainer to avoid human errors. Sections were incubated with a mouse monoclonal antibody against human TWSG1 (Abnova Corporation, Taipei, Taiwan) diluted 1:25 in tris-buffered saline and Tween 20 (TTBS) at 37°C for 32 min.

Manual IHC was optimized from a previous protocol that was obtained from CS lab archive files. A number of optimizations were applied to the previous protocol to maximize BMP4 antigen retrieval on tissue sections and minimize background staining. Enzymatic antigen retrieval with proteinase K appeared to cause over-digestion of the tissue structure, and weak stain signal for BMP4 protein (not

shown). When proteinase K was replaced with heat-induced antigen retrieval by heating 10 mM sodium citrate buffer (pH 6.0) for 15-20 min. Phosphate buffer solution (PBS) was used as washing buffer, which appeared to cause the background to be overstained and also high-surface tension on the slides. PBS was then replaced with TTBS, to reduce the surface tension and the background. The detergent Tween20 is used to reduce the surface tension of the TBS; however, the high concentration of Tween20 can lead to a destruction of the cellular morphology. Unimmunized serum should be applied after antigen retrieval and before primary antibody incubation to block non-specific protein binding. Goat serum is the common blocking serum used for IHC. Blocking serum must be chosen based on the species of the primary antibody and the secondary antibody; wrong selection of blocking serum will result in high background staining. Avoiding dryness of the tissue section is a critical point to perform successful IHC. Therefore, a humidity chamber must be applied during all of the staining process to avoid any drying of tissues. A rabbit polyclonal antibody against human BMP4 (Abcam; Cambridge, Massachusetts, USA) diluted 1:300 with TTBS was used for manual immunostaining.

For the BMP4 studies, sections were deparaffinized in xylene (2X 10 min each) and rehydrated through a series of graded ethanols (2X 100% 10 min each, 1X 95%, 1X 70% and 1X 50% 5 min each), followed by incubation for 20 minutes in 3% hydrogen peroxidase to block the endogenous peroxidase activity. Heating antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) for

15 minutes. Non-immunized goat serum was used to block non-specific protein binding for 60 minutes and sections were incubated overnight at 4°C with rabbit primary antibody to BMP4. Tissue sections were given three washes 5 minutes each with TTBS and incubated with the secondary antibody, biotinylated goat anti-rabbit immunoglobulin (IgG) for 60 minutes before incubating with Avidin-Biotin Complex (ABC) (Vector Laboratories, Burlington, ON, Canada) for 30 minutes. Avidin binds several biotinylated reporter enzyme molecules to form the ABC prior to binding the biotinlyated secondary antibody, thereby amplifying its detection. The antibody complex was visualized as a brown color with DAB Peroxidase Substrate (Dako, Carpinteria, CA, USA) and tissue sections were counterstained with Harris hematoxylin (Thermo Fisher Scientific Anatomical Pathology, Ottawa, ON, Canada). Negative controls were used (absence of primary antibody).

Immunostaining results were evaluated, graded and scored by extent (0=none, 1=1-25%, 22=26-50%, 3=51-75%, 4=76-100% of the tumor cells) and intensity (0=negative, 1=weak, 2=moderate, and 3=intense staining). An immunohistochemical score was calculated for each case in which the extent (percentage of positive stain rating) was multiplied by the intensity rating according to a method validated previously for non-parametric evaluations (Sergi *et al*., 2000). For instance, the extent of the tumor cells 4, multiplied by the intensity of the staining protein 3, results in an immunohistochemical score of 12 (4*3 = 12). In each specimen, tumor cells were randomly selected and counted.

For double-immunostaining, this same method was applied where the first primary antibody was TWSG1 (1:200) and then the section was reheated with 10 mM sodium citrate buffer (pH 6.0) for 5 min to retrieve antigen before applying the second primary antibody, mouse monoclonal BMP4 against human (1:100) (Abcam, Cambridge, USA). Slides then visualized with the second stain V.I.P Peroxidase substrate (Vector, CA, USA).

Statistical analysis

The total number of cells counted in a determinate structure and the results of the percentages of positive cells were presented as mean ± standard deviation (SD). The Mann-Whitney U test was used to test two groups with paired data with non-parametric distribution. All p values were two-tailed and p values <0.05 were considered to indicate a statistically significant difference. Reliability of intra-observer scores (reproducibility or agreement of one observer's or evaluator's score of the same tissue at different times), and inter-observer scores (reproducibility or agreement of scores of the same tissue from different observers or evaluators) was calculated as [Agreements (A) and disagreements (D)] (A/A+D*100). Discrepancies by one evaluator, and discrepancies between the observers were <5% and non-concordant cases were re-evaluated simultaneously by three observers (JJ, BC and CS). The statistical software used was SPSS V.20 (IBM, Armonk, NY, USA).

2.2.4 Cell lines

Human CCA cell lines, including OZ (JCRB1032), HuH28 (JCRB0426), and

HuCCT1 (JCRB0425) were obtained from the cell culture bank of the Japan Health Sciences Foundation (Tokyo, Japan). Hence, OZ and HuH28 cell lines were established from two patients with a poorly differentiated tumor (G3) (See section 1.5.2). HuCCT1 was established from a patient with a moderately differentiated tumor (G2) (See section 1.5.2) (Homma *et al*., 1987; Kusaka *et al*., 1988). HuCCT1 and HuH28 were grown as monolayer cultures in Roswell Park Memorial Institute (RPMI) 1680 medium (Invitrogen Canada Inc. Burlington, ON, Canada). OZ was grown as monolayer cultures in William E medium (Invitrogen Canada Inc. Burlington, ON, Canada). All media were supplemented with 10% fetal bovine serum (FBS) (PAA laboratories Inc., Etobicoke, ON, Canada) and 50 μg/ml gentamicin sulfate (Invitrogen Canada Inc. Burlington, ON, Canada) and incubated at 37°C with 5% CO_2 in a humidified incubation chamber. Human HCC cell line HepG2 (ATCC® HB-8065) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA), and HuH-7 (JCRB0403) was purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. HepG2 and HuH-7 were established from patients with well-differentiated tumors (G1) (See section 1.5.1) (Aden *et al*., 1979; Nakabayashi *et al*., 1982). HCC cell lines were grown as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM), (Invitrogen Canada Inc. Burlington, ON, Canada) supplemented with 10% FBS, 50 μg/ml gentamicin sulfate and incubated at 37°C with 5% CO_2 in a humidified incubation chamber.

An immortalized normal human liver cell line “Tissue Hepatic Liver Epithelial”

(THLE-3) (ATCC® CRL-11233™) was purchased from the ATCC. The cells were maintained in pre-coated flasks with a mixture of bronectin (0.01 mg/mL), bovine collagen type 1 (0.03 mg/ mL), and bovine serum albumin (BSA) (0.01 mg/mL) dissolved in bronchial tracheal epithelial cell growth (BEGM) medium (Invitrogen Canada Inc. Burlington, ON, Canada), and incubated at 37°C and 5% CO_2.

2.2.5 Western blot technique

Cells were harvested and lysed in radio-immuno precipitation assay (RIPA) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) lysis buffer. Briefly, cells were washed three times with PBS and then incubated with RIPA lysis buffer and protease inhibitor (to inhibit protein degradation by endogenous proteases) on ice for 5-10 min. Cells were gently scraped and collected in microtubes. Lysed cells were sonicated at 20-50 kHz (Sonic Dismembrator model 500, Fisher Scientific) for few seconds and then centrifuged for 10 minutes at 14,000 x g in a cold (4°C) microfuge (Millipore, Toronto, ON, Canada). The bicinchoninic acid (BCA) assay (Fisher Scientific Company, Ottawa, ON, Canada) was used for the colorimetric detection and quantitation of total extracted protein. Briefly, protein sample concentrations are determined by comparing their absorbance to that of a dilution-series of a BSA standard. Both protein samples and BSA standards are mixed with assay reagent and their absorbances measured by using a spectrophotometer. The absorbance of each standard is plotted versus its concentration to generate a standard curve. The sample protein concentrations are then read from the standard curve.

The Bio-Rad equipment manual protocol from was used for SDS-PAGE gel preparation and the Western blot technique (www.bio-rad.com). Sample proteins are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Inc., Baie d'Urfe, Quebec, Canada). Fellow graduate student, Yasser Abuetabh, assisted with the Western Blot studies.

Briefly, SDS-PAGE is consists of two gels, a stacking gel where the protein samples are loaded and a resolving gel where the protein samples separate. Nine percent resolving gel is prepared by mixing a 6.75 ml of H_2O with 4.5 ml of 44.4% acrylamide, 100 µl 10% ammonium persulfate and 3.75 ml of 10% SDS 6.8 glycine buffer and then 10 µl of Temed to solidify this mixture, and promptly but carefully poured to avoid the formation of bubbles. The stacking gel was prepared by adding 3.05 ml of H_2O to 650 µl of 44.4% acrylamide, 25 µl 10% ammonium persulfate and 1.250 ml of 10% SDS in pH 6.8 glycine buffer and then mixed with 8 µl of Temed to solidify this mixture and promptly but carefully poured on top of the resolving gel. The sample well comb was immediately inserted.

Samples were boiled for 5 minutes and loaded into each well. Samples were run on 9% polyacrylamide gels in Tris-glycine running buffer (24.9 mM Tris-base, 192 mM glycine, 0.1% SDS) at 140 V for 45-60 minutes using the Mini-Trans-

Blot® (Bio-Rad Laboratories, Mississauga ON). PVDF membranes and gels were incubated in transfer buffer (25 mM Tris-base, 192 mM glycine, 0.05%, 20% methanol) (Sigma Aldrich) for 3-5 min. Proteins were transferred onto PVDF membranes at 140 V for 60 min in a cold room (4°C) using the Mini-Trans-Blot®. After an hour blocking with 5% non-fat dry milk at room temperature, the membranes were incubated with mouse monoclonal primary antibodies against human TWSG1 (1:500) (Abnova Corporation, Taipei, Taiwan), a rabbit polyclonal antibody against human BMP4 (1:1000) (Abcam; Cambridge, Massachusetts, USA) and β-actin (1:5000) (Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4°C; all antibodies are diluted with 5% non-fat milk in TTBS. The membranes were then incubated with secondary antibody; horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG for BMP4 and goat anti-mouse IgG for TWSG1 and goat anti-mouse IgG β-actin for 60 minutes at room temperature (GE Healthcare Inc., Baie d'Urfe, Quebec, Canada). Detection was performed with an enhanced chemiluminescent (ECL) substrate by incubating the membranes with ECL exposed to the light for 3 min (Perkin-Elmer Inc., Waltham, MA, USA) and then exposed to Kodak X-ray film in the dark room for 3-5 min (Kodak Graphic Communications Company, Burnaby, BC, Canada). All experiments were performed in triplicate (n=3).

2.3 Results

The demographic characteristics of the cases selected for the microarray analysis are shown in (Table 2.1). Overall, TWSG1 in CCA showed a stronger and more diffuse immunohistochemical signal than HCC (Figure 2.2 and 2.3). With regard

to CCA, in the group with G2 or intermediate differentiation, three out of seven samples had a score of 12, one sample had a score of 8 and two samples had a score of 4 while, in the group with G3 or poorly-differentiated carcinoma, one out of five samples had a score of 12 and four samples had a score of 4 (Figure 2.4, 2.5). HCC with G1-G2 degrees of differentiation (well- and moderately-differentiated) had a score of 0 in nine out of 10 cases and one sample had a score of 8. Among HCC tumors with G3-G4 degrees of differentiation (poorly-differentiated or undifferentiated), one sample had a score of 8 and two had a score of 0 (Figure 2.5, 2.6). For TWSG1, CCA had a mean score of 4.5 (±2.1 SD), while HCC had a mean score of 2.0 (±1.6 SD); this difference was significant ($p = 0.004$; Figure 2.4). Interestingly, the expression was particularly intense in the areas close to areas with desmoplastic stroma and glandular differentiation (Figure 2.2 A-C). Non-neoplastic hepatocytes or surrounding liver tissue had no or minimal background staining.

Western blot technique detected TWSG1 in all three CCA cell lines, although the intensity was stronger in OZ than in HuH28 and HuCCT1 (Figure 2.7). Normal hepatocytes in culture (THLE-3) showed no expression. HepG2 had a band of strong intensity. The β-actin signal was present at comparable levels in all cell lines examined.

BMP4 was detected in CCA (Figure 2.8 A, B) and partly in HCC (Figure 2.8 C, D) by immunohistochemistry. Although only 13 cases (5 CCA and 8 HCC) have

been investigated for BMP4 expression because of the tissue core loss during microtome cutting, all five CCA cases tested showed intense and quite diffuse staining, while only one of eight HCC cases tested showed positive staining. With regard to CCA, in the group with G2 or intermediate differentiation, one sample had a score of 12, and one sample had a score of 6 while, in the group with G3 or poorly-differentiated carcinoma, one out of three samples had a score of 12 and two samples had a score of 4 (Figure 2.9, 2.10). HCC with G1-G2 degrees of differentiation (well- and moderately-differentiated) had a score of 0 in five out of six cases and one sample had a score of 4. In HCC with G3 degrees of differentiation (poorly-differentiated), only one sample had a score of 0 (Figure 2.10, 2.11). Only one case had strong endothelial expression of BMP4, which was then used as internal positive control for both CCA and HCC (Figure 2.8 D). For BMP4, CCA had a mean score of 7.6 (±4.1 SD), while HCC had a mean score of 0.5 (±4.4 SD); this difference was statistically significant ($p = 0.02$; Figure 2.9).

Western blot technique detected BMP4 in CCA and HCC cell lines, although the strongest intensity was detected in HuH-7 cell line (Figure 2.12).

Double immunostaining showed no co-localization between BMP4 and TWSG1 in HCC and CCA tumors. TWSG1 expression was located at the epithelial and stromal cells, whereas BMP4 expression was mainly located at the stromal cells (Figure 2.13).

2.4 Discussion

Many developmentally important signaling pathways and proteins are reactivated or differentially regulated during oncogenesis. This study demonstrates the presence of TWSG1 in liver tumors, suggesting that components of the BMP signaling pathway may be reactivated. A strong intensity of immunostaining was correlated with tumor invasiveness (loss of cell differentiation), but more extensive studies are warranted because sample size hindered the statistical analysis.

Liver cancer is of epithelial origin, so epithelial and glandular staining is significant in representing the presence of TWSG1 at the site of the cancer. Interestingly, CCA had strong TWSG1 immunostaining, which was present in areas of desmoplastic stroma and glandular differentiation of HCC (Figure 2.2 A-C). This points to a possible association of TWSG1 with glandular differentiation of carcinomas. Enriched immunostaining for TWSG1 during the invasive phases of gland-forming epithelial tumors raises questions about a possible role for TWSG1 in the cytoskeleton remodeling and adhesion, modulating invasive behavior or initiating the process of lymphovascular invasion.

There was no correlation between TWSG1 and BMP4 expression in CCA or HCC. Only one case showed BMP4 positivity in HCC even though the endothelial cells, which served as an internal control, were positive in both HCC and CCA (Figure 2.8 D). Double immunostaining is not an ideal approach to

detect the co-localization between the two proteins in this study and limits the interpretation of this study. Further investigations should explore the relationship between TWSG1 and BMP4 in liver tumors and their role in the pathogenesis of cancer (Deng *et al*., 2007;Maegdefrau *et al*., 2009;Quante *et al*., 2011).

The relationship of TWSG1 with BMP4 is intriguing. The interplay of several signaling pathways that provide positional information and induce cell fate specification controls the development of tissue and organs in multicellular organisms as well as multiple steps of carcinogenesis in epithelial cells of liver tumors, both *in vitro* and *in vivo*.

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Table 2.1. Demographic characteristics of tumor cases selected for tissue microarray.

Proteins	**TWSG1**		**BMP4**	
Diagnosis	**CCA**	**HCC**	**CCA**	**HCC**
Total cases	12	13	5	8
Male	5	10	2	5
Female	7	3	3	3
Age range	41-77	30-77	41-77	30-77
G1	0	4	0	1
G2	7	5	2	5
G2-G3	1	1	1	0
G3	4	3	2	2

G1, low grade or well-differentiated; G2, intermediate grade or moderately-differentiated; G3, high grade or poorly-differentiated. CCA, cholangiocellular carcinoma; HCC, hepatocellular carcinoma. (See Table 1.1 for HCC tumor grading, and Table 1.3 for CCA tumor grading)

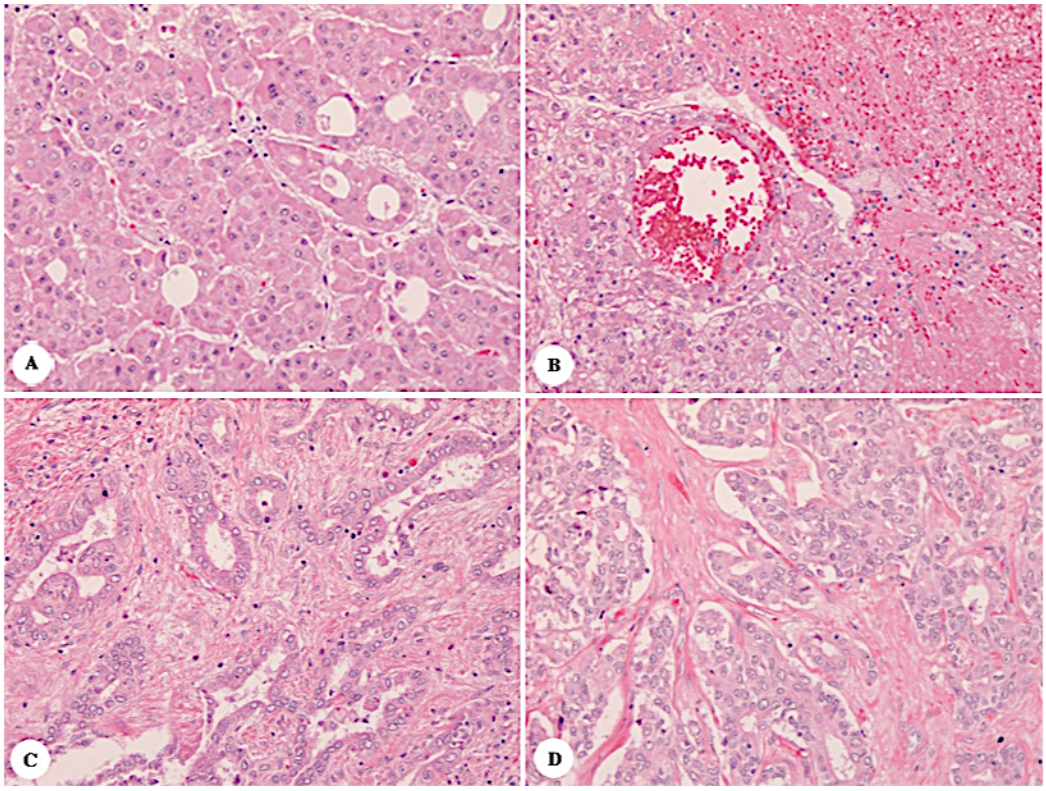

Figure 2.1. H & E stain for hepatocellular carcinoma (HCC) (A-B), and cholangiocellular carcinoma (C-D). (A-D, 200X).

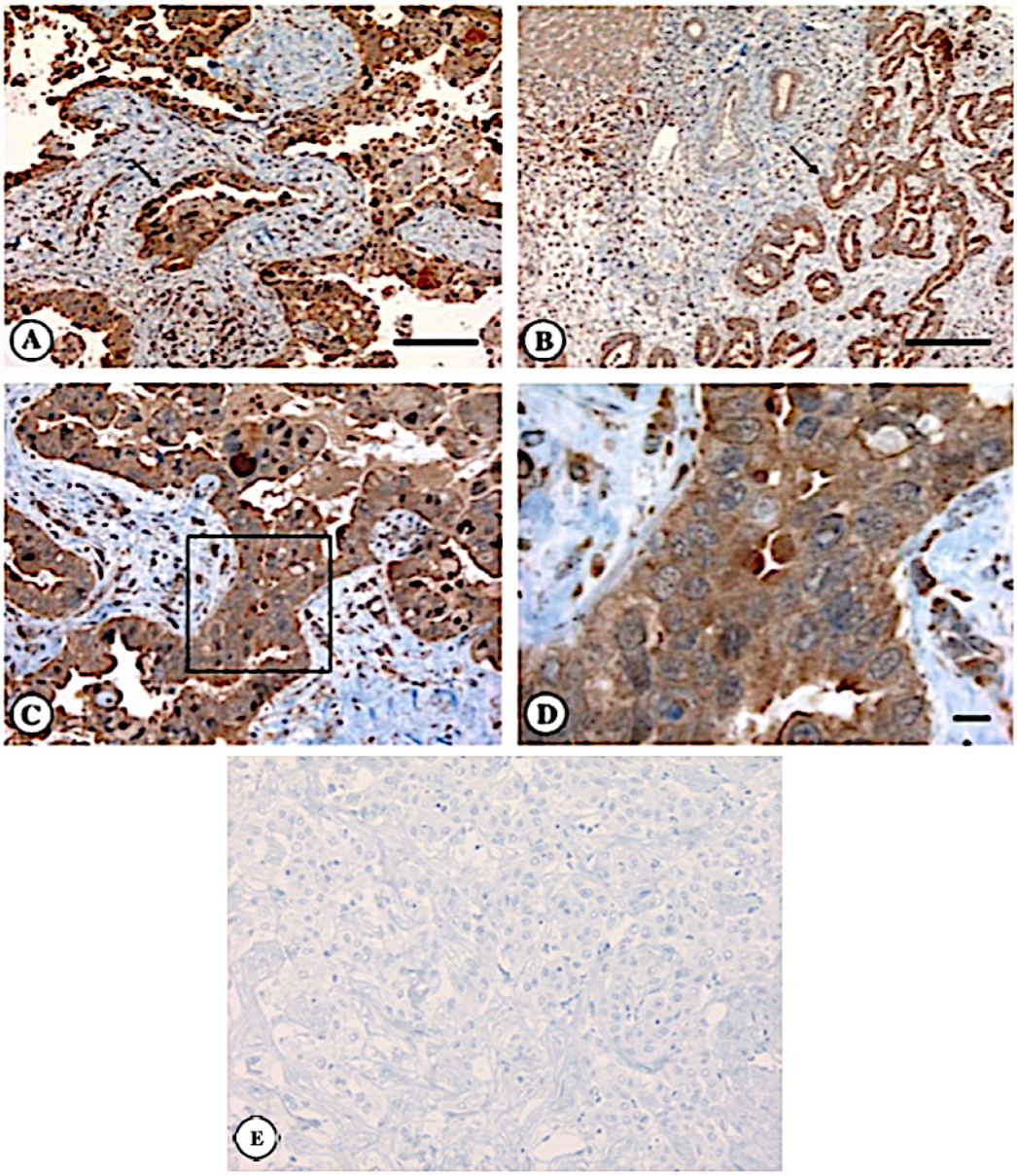

Figure 2.2. (A-D) Immunolocalization of Twisted gastrulation (TWSG1) in cholangiocellular carcinoma (CCA). (Brown) TWSG1 is strongly expressed in the malignant glands of CCA with a strong signal (A-C) in the cytoplasm of malignant glandular component surrounded by stroma desmoplasia, which is characteristic of CCA (arrows). (D) Highlighted area of interest of (C) showing the predominant cytoplasmic signaling. (E) Negative control. (A, 100X, scale bar= 200 μm; B, 100X, scale bar=200 μm; C, 200X; D, 630X, scale bar=10 μm; E, 200X).

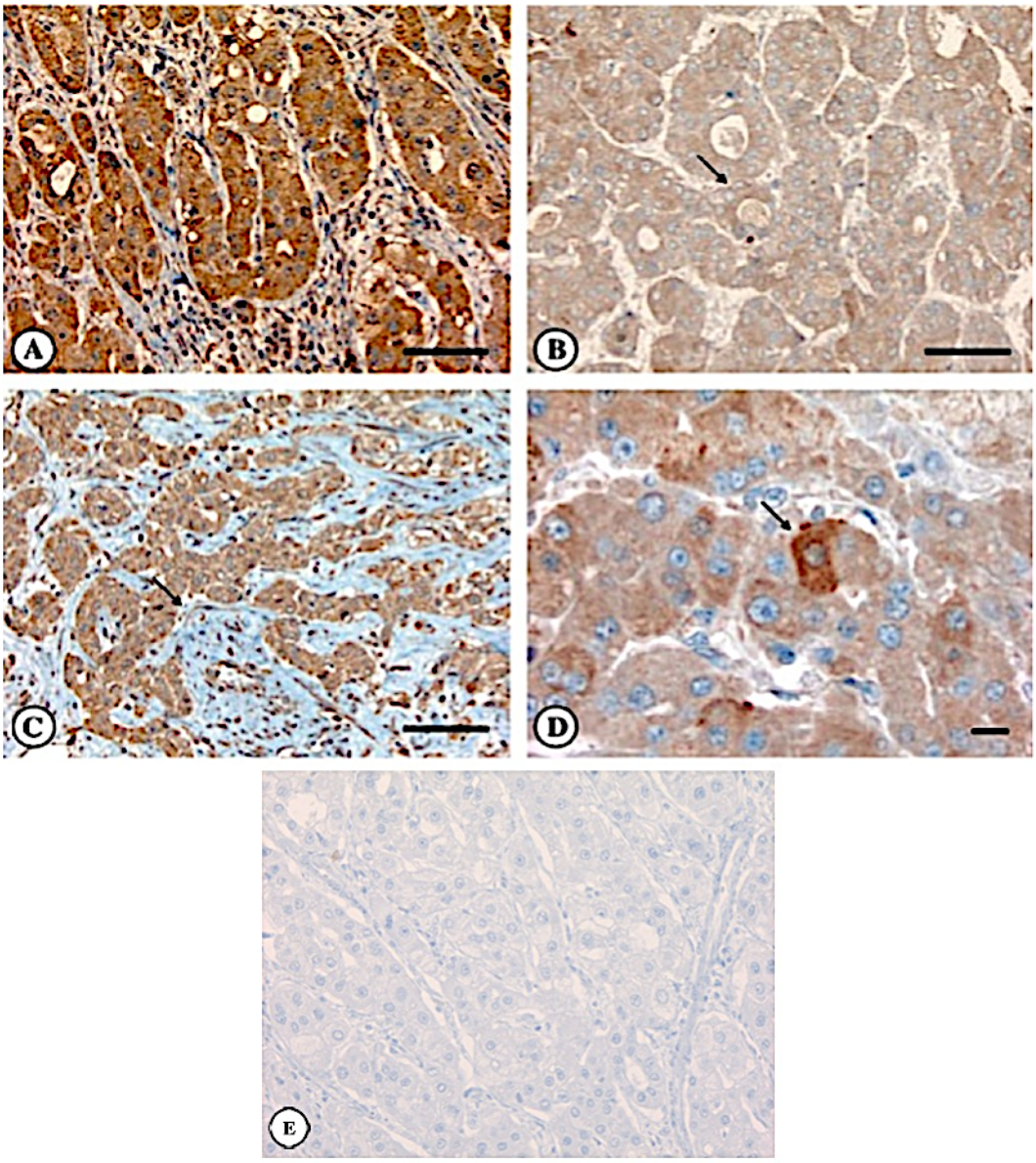

Figure 2.3. Immunolocalization of Twisted gastrulation (TWSG1) in hepatocellular carcinoma (HCC) (A-D). (Brown) strong (A) to slight or no expression (B-D) was seen in HCC, with more intensity detected in the gland forming tumor components (arrow in B). The arrow in 2C indicates the interface between the epithelial and myoepithelial compartments. 2D (arrow) shows a single malignant cell of a HCC trabecular variant with accentuated staining. (E) Negative control. (A, 200X, scale bar=10 μm; B, 200X, scale bar=10 μm; C, 200X, scale bar=10 μm; D, 630X, scale bar=10 μm; E, 200X).

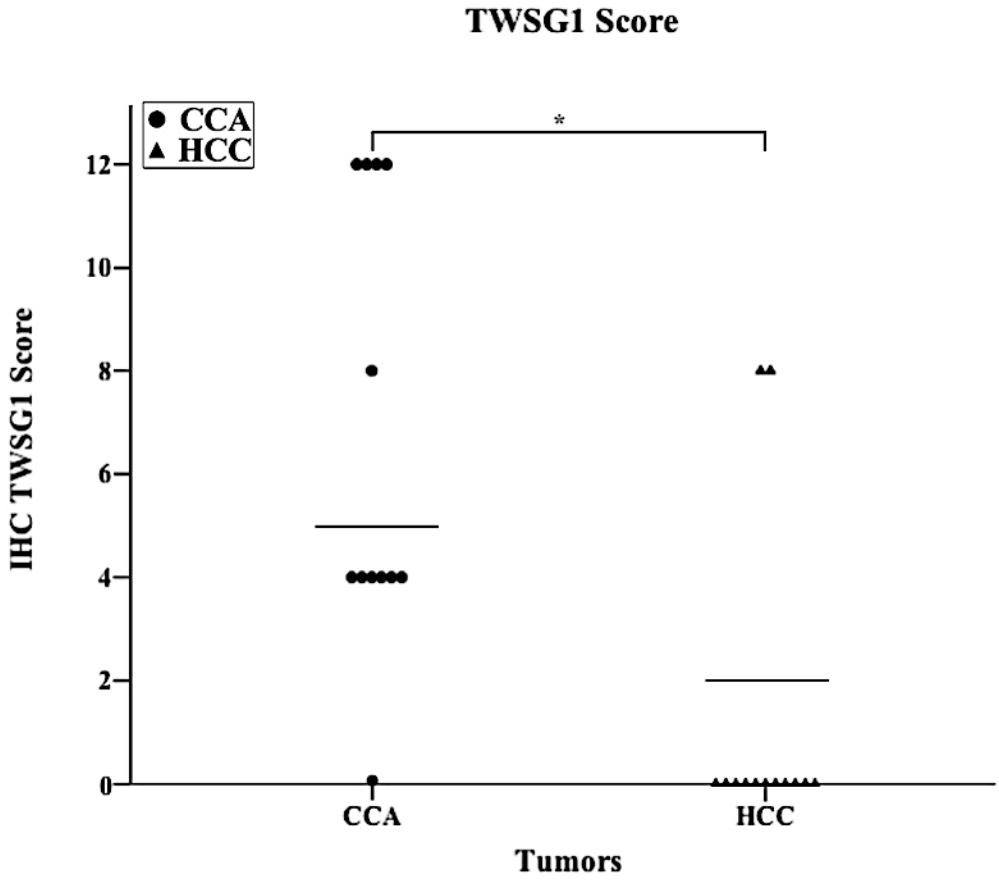

Figure 2.4. Twisted gastrulation (TWSG)-1 score in cholangiocellular carcinoma (CCA) and hepatocellular carcinoma (HCC). *p=0.004

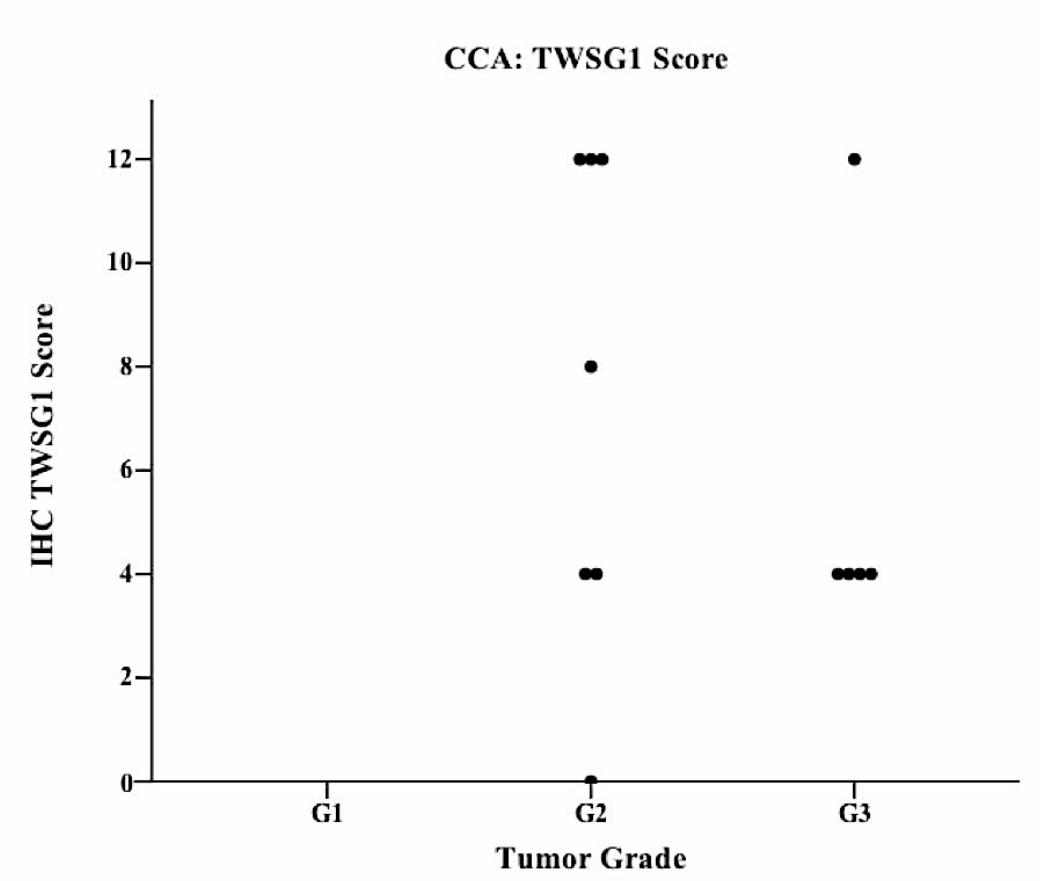

Figure 2.5. Twisted gastrulation protein (TWSG)-1 IHC score and tumor grade of cholangiocellular carcinoma (CCA).

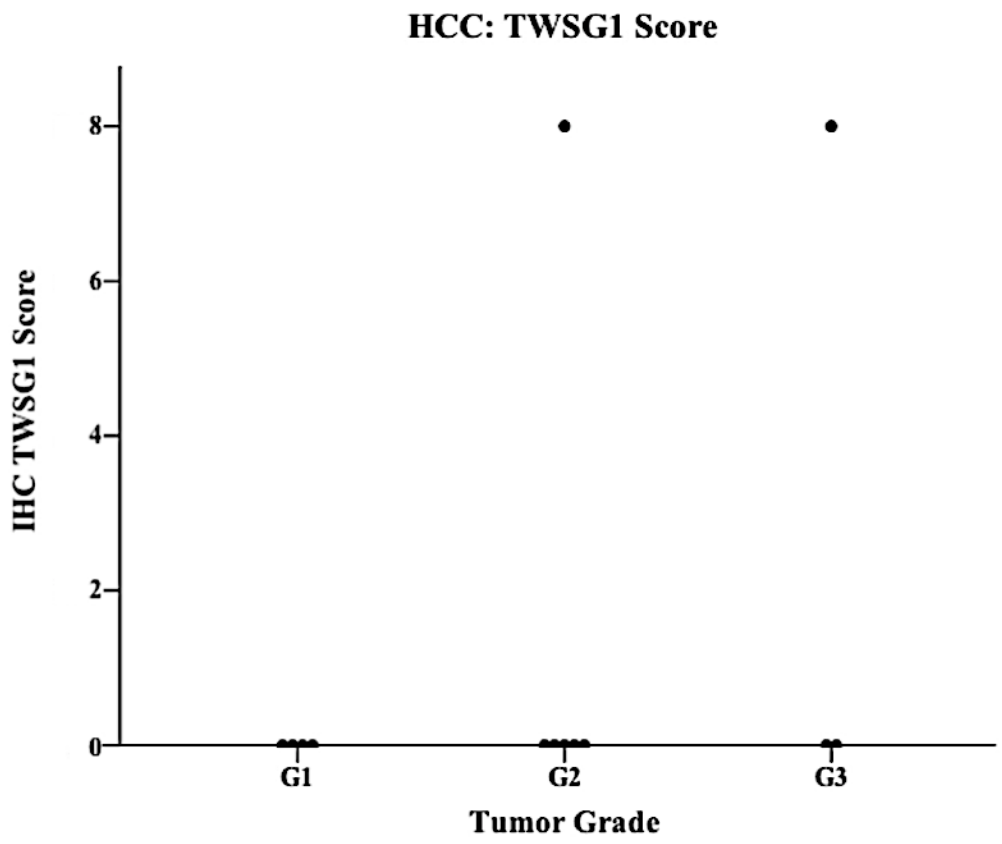

Figure 2.6. Twisted gastrulation protein (TWSG)-1 IHC score and tumor grade of hepatocellular carcinoma (HCC).

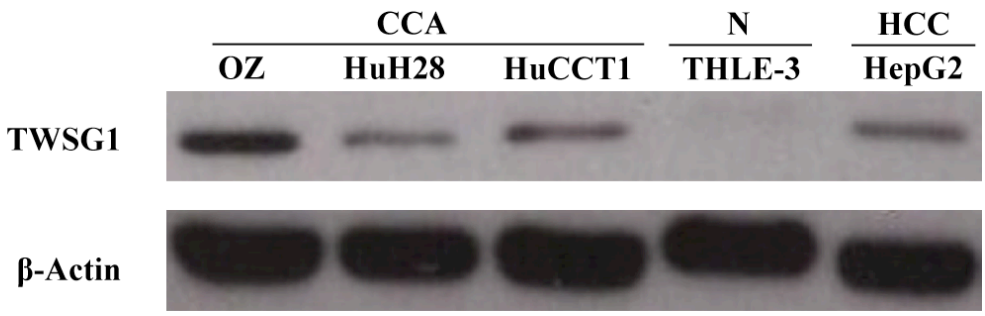

Figure 2.7. Western blot technique of Twisted gastrulation (TWSG1) expression in cholangiocellular carcinoma cell lines (OZ, HuH28 and HuCCT1), Papovavirus-immortalized normal hepatocytes (THLE-3) and hepatocellular carcinoma cell line (HepG2). β-actin detection was used as loading control for constitutive protein expression to ensure consistent loading in each gel well.

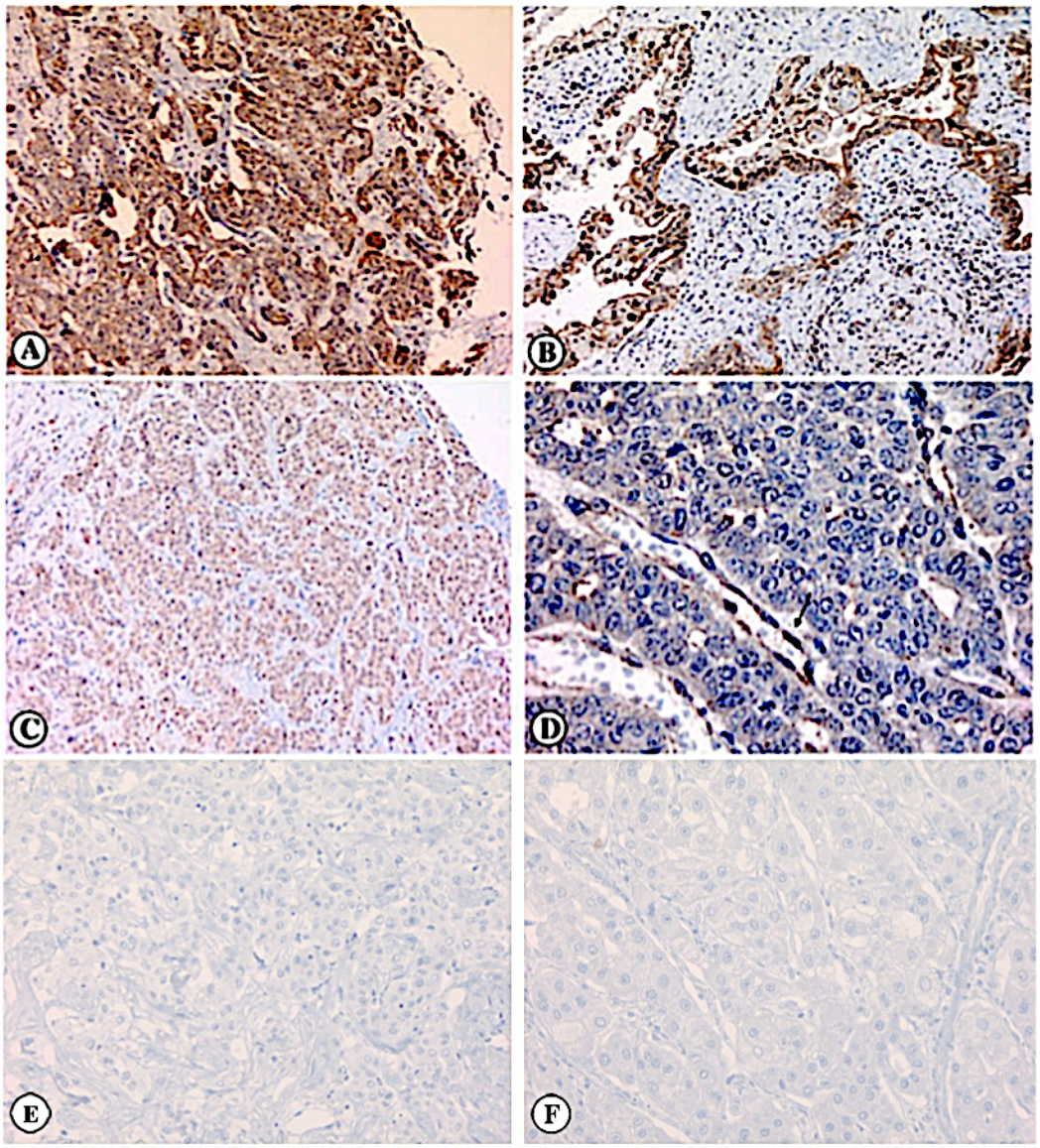

Figure 2.8. (A-D) Immunolocalization of the bone morphogenetic protein BMP4 in cholangiocellular carcinoma (CCA) and hepatocellular carcinoma (HCC). BMP4 is seen in two CCA cases (A, B) and one HCC case (C), with no staining in a HCC macrotrabecular variant (D). In (D) the arrow shows the endothelial cells, which are positive and have been used as a positive internal control for BMP4 expression. (E and F) Negative controls for CCA and HCC, respectively. (A, 200X; B, 200X; C, 200X; D, 400X; E, 200X; F, 200X).

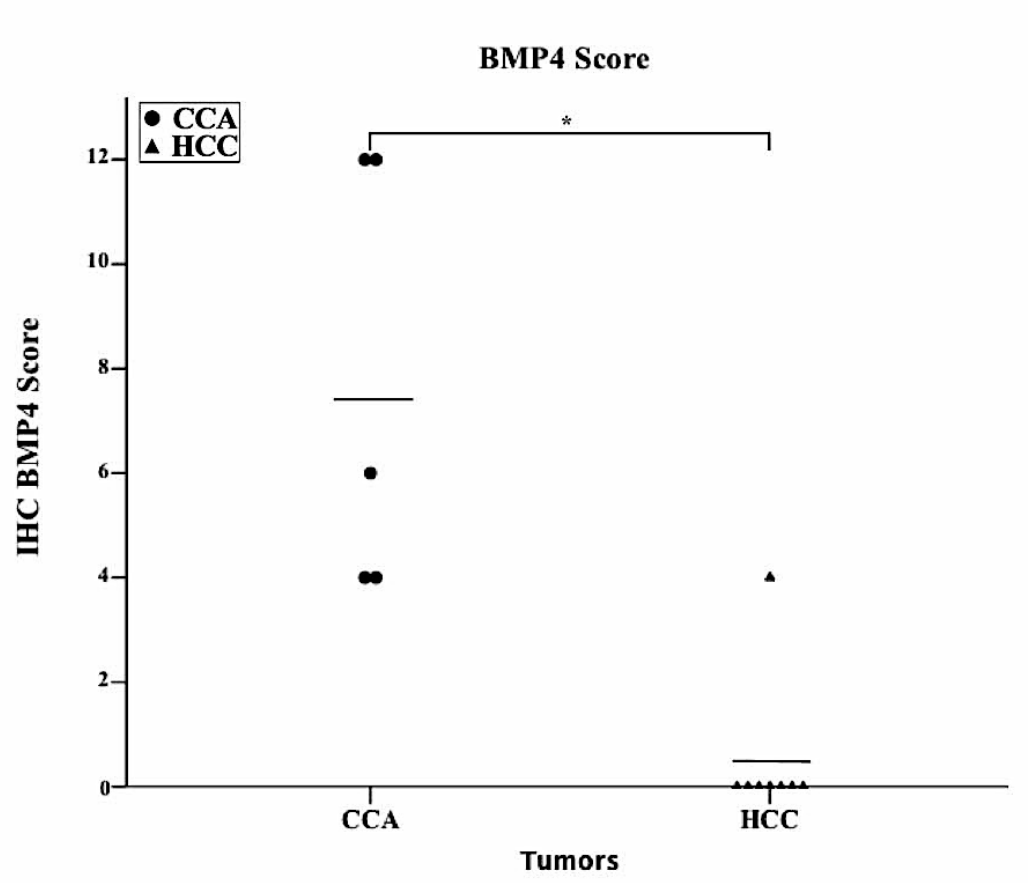

Figure 2.9. Bone morphogenetic protein (BMP)-4 IHC score in cholangiocellular carcinoma (CCA) and hepatocellular carcinoma (HCC). *p=0.02

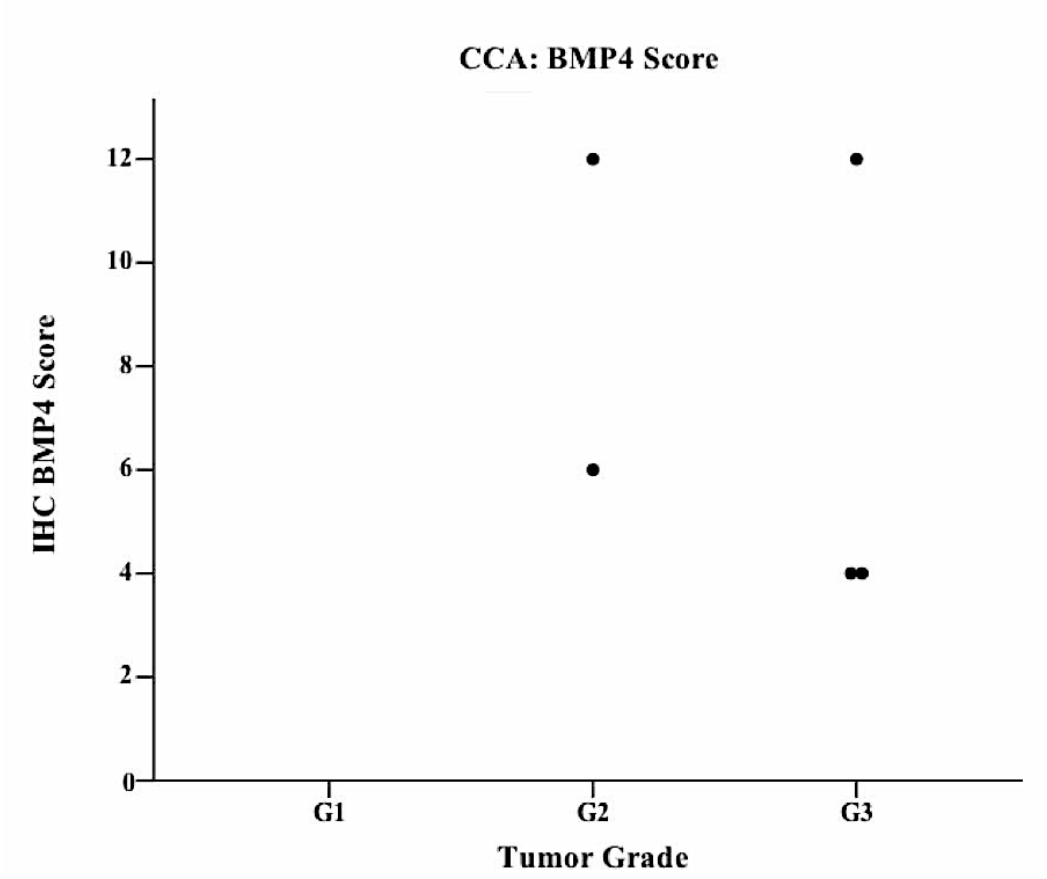

Figure 2.10. Bone morphogenetic protein (BMP)-4 IHC score and tumor grade of cholangiocellular carcinoma (CCA).

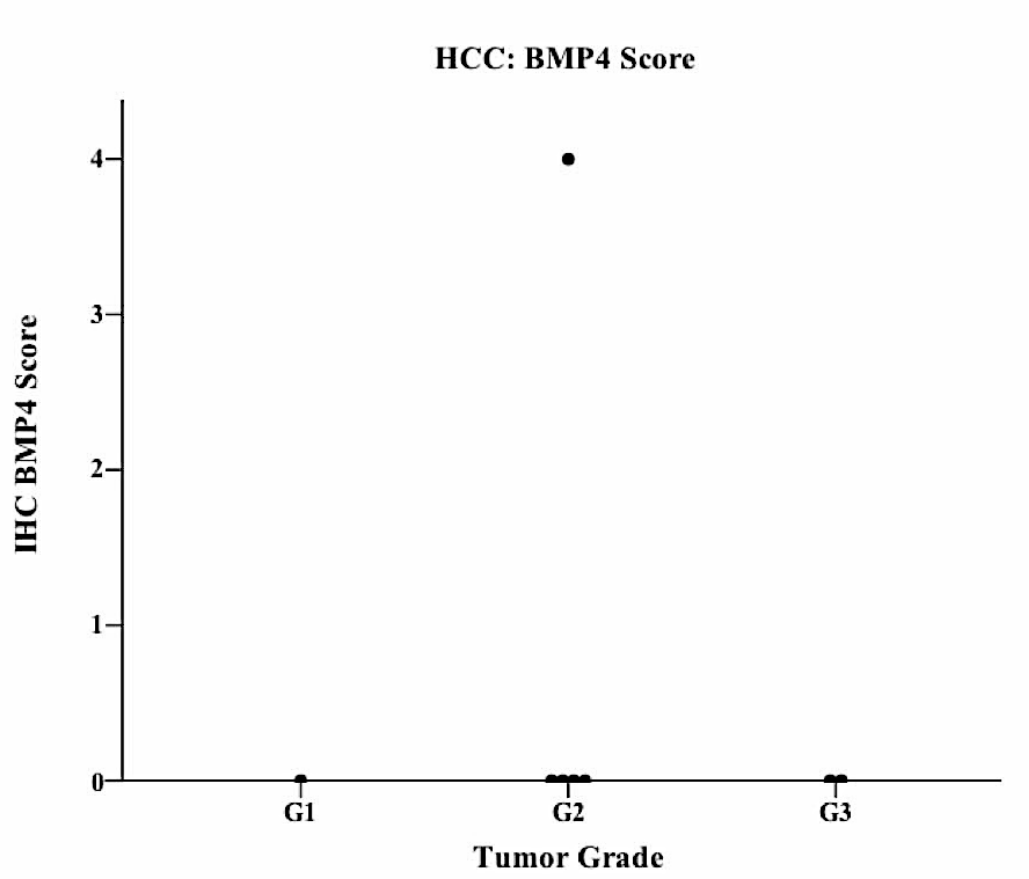

Figure 2.11. Bone morphogenetic protein (BMP)-4 IHC score and tumor grade of hepatocellular carcinoma (HCC).

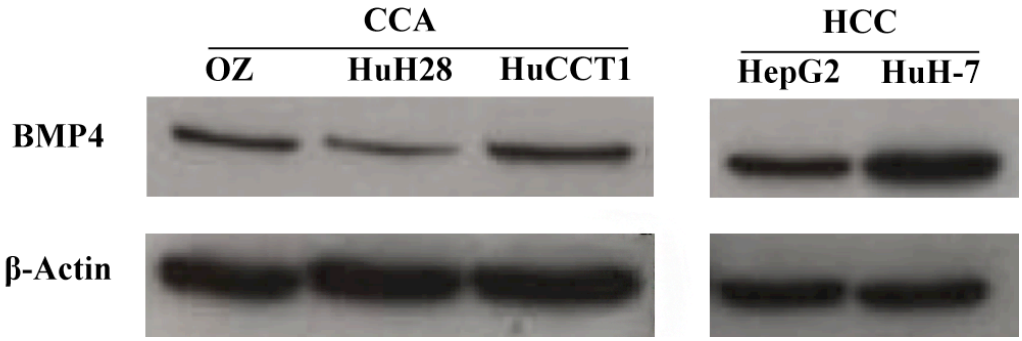

Figure 2.12. Western blot technique of the bone morphogenetic protein (BMP)-4 expression in cholangiocellular carcinoma cell lines (OZ, HuH28 and HuCCT1), Hepatocellular carcinoma cell lines (HepG2 and HuH-7). β-actin detection was used as loading control for constitutive protein expression to ensure consistent loading in each gel well.

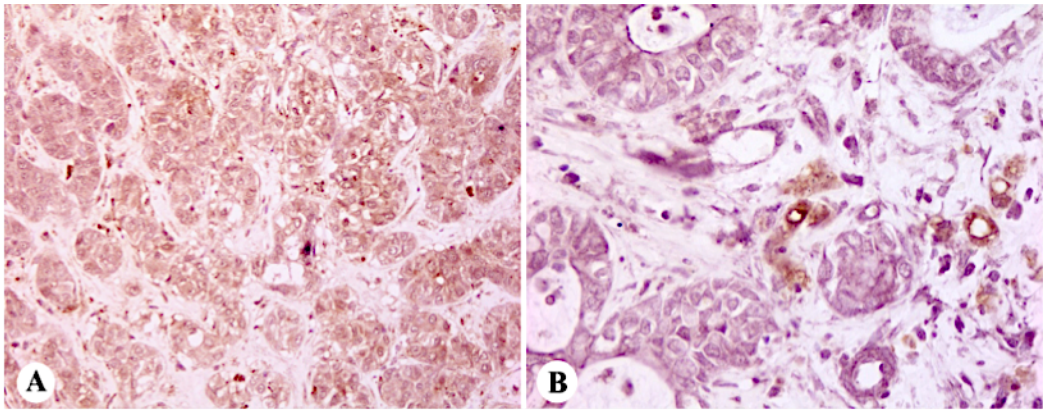

Figure 2.13. Co-localization immunohistochemistry double stain of Twisted gastrulation (TWSG1) purple color and Bone morphgentic protein (BMP)-4 brown color (A) in Hepatocellular carcinoma (HCC) and (B) in Cholangiocellular carcinoma (CCA). (A, 200X; B, 400X)

Chapter 3

Differential expression of sonic hedgehog in human hepatocellular carcinoma and cholangiocellular carcinoma

A version of this chapter has been submitted for publication to *Human Pathology*

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Differential expression of sonic hedgehog in human hepatocellular carcinoma and cholangiocellular carcinoma.

3.1 Introduction

Hepatocellular carcinoma (HCC) is an aggressive primary liver tumor that arises from hepatocytes, the major cell type in the liver (Blonski *et al*., 2010). HCC accounts for 80%-90% of primary liver cancer and represents the fifth most common cancer worldwide (Blonski *et al*., 2010). Due to its high mortality rate, HCC is the third most fatal malignant neoplasm, killing more than 500,000 people annually (Parkin *et al*., 2005). HCC has a poor prognosis with a five-year survival rate of less than 5%, and usually diagnosed at an advanced stage or when the diameter of the tumor exceeds 10 cm (Motola-Kuba *et al*., 2006). The pathophysiological process detected in more than 80% of HCC cases involves cirrhosis caused by Hepatitis B and C viruses (Motola-Kuba *et al*., 2006). On the other hand, cholangiocellular carcinoma (CCA) is a malignant neoplasm that arises from the epithelium of the biliary tract (Khan *et al*., 2005). After HCC, CCA represents the second most serious primary liver cancer worldwide (Khan *et al*., 2005). The incidence of CCA has been strongly linked to chronic inflammation of the biliary tract, resulting from several risk factors. Specifically, the highest mortality rate was reported in East Asia, especially in Korea and Northeast Thailand, and was caused by liver fluke infestations such as *Clonorchis sinensis* and *Opisthorchis viverrini*, as well as primary sclerosis cholangitis (PSC) (de Martel *et al*., 2010; Olnes and Erlich, 2004).

In 1978, the hedgehog (Hh) gene was initially identified during a *Drosophila* mutagenesis screen (Nusslein-Volhard and Wieschaus, 1980). The mutated Hh

gene possessed the appearance of a hedgehog, corresponding to small projections covering the surface of the fruit fly embryo (Nusslein-Volhard and Wieschaus, 1980). Moreover, Hh genes play a central role in mammalian development, including cell proliferation, tissue polarity (Hirose *et al*., 2009; Litingtung *et al*., 1998)

Sonic hedgehog (Shh) is a secreted glycoprotein member of the Hh family that regulates the anterior/posterior patterning in limb development, the polarity in the central nervous system, and many types of cell differentiation (Dessaud *et al*., 2008). Briefly, Shh interacts with its receptor patched (Ptch)-1, which activates the hedgehog gene target. In the absence of Shh ligand, Ptch-1 represses the 7-transmembrane protein smoothened (SMO), which inactivates the Shh signaling pathway (Dessaud *et al*., 2008) (see chapter 1.2). Holoprosencephaly is a frontal brain defect caused by a mutation or dysregulation of Shh ligand during embryogenesis (Gekas *et al*., 2012). Studies have reported an upregulation of Shh ligand, as well as mutations in Ptch-1 and SMO, are often present in cancer cells such as nevoid basal cell carcinoma, primitive neuroectodermal tumors and colorectal carcinoma (Hahn *et al*., 1996; Reifenberger *et al*., 1998; Yoshikawa *et al*., 2009).

The transforming growth factor beta (TGF-β) superfamily regulates both human and animal embryonic development (Shen, 2007; Zorn, 2008). Accordingly, the failure or dysregulation of this superfamily has been involved in different types of

diseases, including carcinogenesis (Fan *et al*., 2010; Regateiro *et al*., 2011). Bone morphogenic protein (BMP)-4 is one of the TGF-β isoforms that plays an important role during embryonic development events such as the proliferation, differentiation, and morphogenesis of organs (Liem *et al*., 1995). In addition, this protein performs a vital role in dorsal-ventral formation of the neural tube during embryogenesis, as well as bone formation (Liem *et al*., 1995). Moreover, studies have reported that BMP4 has a diverse role during carcinogenesis, potentially functioning as a promoter of cell migration or as a tumor inhibitor (Alarmo *et al*., 2013). Recently, a study by Wang *et al*., focusing on carcinogenesis in Barrett's esophagus, suggested that Shh becomes reactivated through gastric acid and bile injury, inducing the stromal BMP4, which subsequently reprograms the epithelial cells of the esophagus to favor a columnar phenotype (Wang *et al*., 2010).

Hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCA) are the two most common primary adult liver malignancies, and the molecular mechanisms underlying the development of both conditions are still poorly understood. The present study seeks to understand the role of the Shh signaling pathway in carcinogenesis and/or the progression of HCC and CCA. Accordingly, the aim of this study was to investigate the expression of Shh in HCC and CCA.

3.2 Materials and methods:

3.2.1 Tissue samples

Twenty-one tissue specimens of hepatocellular carcinoma (HCC) and 19 tissue specimens of cholangiocellular carcinoma (CCA) were obtained from archive

files of the (Institute of Pathology, University of Heidelberg, Germany) following Ethics Committee approvals. This research is linked to two Human Research Ethics Board approvals (Pro00007657_Molecular Pathology and Genetics of the Abnormalities of the Intrahepatic Biliary System and Pro00020274_Twsg-1 Expression in Cancer). All of the samples were fixed in 4% buffered formaldehyde and routinely processed (See section 2.2.1). Paraffin tissue blocks were sectioned at five μm and each section was stained with hematoxylin and eosin (H&E) Figure (3.1) (See method in section 2.2.2). All cases were retrospective and were anonymized by the principle investigator. Consecutive sections were used for immunohistochemistry.

3.2.2 Cell culture

CCA cell lines, including OZ, HuH28, and HuCCT1 were obtained from the cell culture bank of the Japan Health Sciences Foundation (See section 2.2.4). All cell lines were grown as monolayer cultures in their appropriate media as previously described (Abuetabh *et al*., 2011) (See section 2.2.4). Furthermore, human HCC cell lines were also studied, including HepG2 and HuH-7. HepG2 was purchased from the American Type Cultural Collection (Manassas, VA, USA). HuH-7 was purchased from the cell culture bank of the Japan Health Sciences Foundation (Tokyo, Japan). Cell lines were cultured as described in section 2.2.4.

3.2.3 Immunohistochemistry and scoring

Formalin-fixed and paraffin-embedded tissue samples were used in this study. Sections were cut at 5-6 μm. Sections were deparaffinized in xylene and

rehydrated through a series of graded ethanol, followed by 3% hydrogen peroxidase to block the endogenous peroxidase activity (see section 2.2.3). Antigen retrieval was performed by heating in 10 mM sodium citrate buffer (pH 6.0) for 15 min, followed by blocking with non-immunized goat serum for 60 min. Sections were incubated overnight at 4°C with Shh primary rabbit polyclonal antibodies "(Abcam, Cambridge, MA, USA) diluted 1:200 in tris-buffered saline and Tween 20 (TTBS). Tissue sections were washed three times in TTBS and incubated with the secondary antibody, biotinylated goat anti-rabbit immunoglobulin (IgG) for 60 minutes before incubating with Avidin-Biotin Complex (ABC) (Vector Laboratories, Burlington, ON, Canada) for 30 min. The antibody complex was visualized with DAB Peroxidase Substrate (Dako, Carpinteria, CA, USA) and tissue sections were counterstained with Harris hematoxylin (Thermo Fisher Scientific Anatomical Pathology, Ottawa, ON, Canada) (see section 2.2.3). The absence of primary antibody Shh was used as a negative control. Normal fetal liver was used as a positive control. Shh stained samples were scored by a semi-quantitative method where extent percentage of the positive rating was multiplied by the intensity rating according to the method validated previously for non-parametric evaluations (Sergi *et al*., 2000). Scores were assigned according to the extent (0=none, 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100% of the tumor cells) and intensity of staining (0/negative, 1/weak, 2/moderate, 3/intense) (Sergi *et al*., 2000) (See section 2.2.3).

For the double immunostaining, only one case of moderately-differentiated tumor (G2) was stained with Shh (as above), then the section was reheated for 5 min to retrieve antigen before applying the second primary antibody, mouse monoclonal BMP4 (1:100) (Abcam, Cambridge, USA). BMP4 was then visualized with V.I.P Peroxidase Substrate (Vector, CA, USA).

3.2.4 Statistical analysis

The tumor cells were randomly selected and counted. The total number of cells counted in a determinate structure and the results of the percentages of positive cells were presented as mean ± SD. The Mann-Whitney U test was used compare two groups with paired data with a non-parametric distribution. Pearson bivariate analysis was used to determine the association between Shh scores and tumor grade. All p values were two-tailed and p values less than 0.05 were considered to indicate statistically significant differences between the two groups. The statistical software used was SPSS Version 20 (IBM, Armonk, NY, USA).

3.2.5 Western blot analysis

Western blotting was performed on proteins separated electrophoretically and transferred from SDS-PAGE onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare Inc., Baie d'Urfe, Quebec, Canada) (see section 2.2.5). After blocking with 5% non-fat dry milk in TTBS for 1 hour, the membranes were incubated with a rabbit polyclonal antibody against Shh (1:500) overnight at 4°C (Abcam, Cambridge, MA, USA). The membranes were then incubated with

horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG secondary antibody for 60 minutes at room temperature. β-actin (1:5000) (Cell Signaling Technology Inc., Danvers, MA, USA) was used as a loading control and incubated with the membrane for 60 min at room temperature. Detection was performed with an enhanced chemiluminescent substrate (ECL) applied on the membrane for 5 min at room temperature (Perkin-Elmer Inc., Waltham, MA, USA) and then exposed to Kodak X-ray film (Kodak Graphic Communications Company, Burnaby, BC, Canada) for 3-5 min. All experiments were performed in triplicate.

3.3 Results

The demographic characteristics of the cases selected for IHC are shown in (Table 3.1). First, Shh expression was investigated in human liver cancer tissues using immunohistochemical staining. In most cases, a lack of Shh expression was observed in normal (non-neoplastic) hepatocytes surrounding the nodules of liver carcinomas in the tissue sections. Varied expression, from absent to strong, was present in the neoplastic areas of HCC (Figure 3.2). The expression of Shh in CCA was stronger and diffuse (Figure 3.3). The epithelial expression of Shh was detected in only 15 HCC cases. Specifically, the epithelial Shh expression was significantly higher in liver carcinomas than in normal hepatocytes. A slight to moderate expression was found in 15 out of 21 cases (71.4%), while a no expression of Shh was detected in six cases (28.5%) of HCC. In the twenty-one HCC cases, the Shh expression was given a score from one to seven based on the strength of its expression. A score of 1 was assigned to five cases out of 21. Furthermore, three cases had a score of 2, one case obtained a score of 4, and two

cases achieved a score of 5. Finally, two cases received a score of 6 and one case obtained score of 7, as seen in (Figure 3.4). In contrast, the immunohistochemical staining of CCA revealed a moderate to strong expression of Shh in the neoplastic areas (subluminal) of the epithelial bile duct tumor (Figure 3.3). For CCA, one case was given score of 4, one case was given score of 6, one case was given score of 9, two cases obtained a score of 7, six cases obtained a score of 10, two cases with score of 11 and six cases with a score of 12 (Figure 3.4). With regards to tumor grades, the expression of Shh in HCC was strongly linked with tumor grade, where this expression dramatically increased from a well-differentiated to a poorly-differentiated tumor ($p = 0.016$), as seen in (Figure 3.5). In contrast, the correlation between Shh expression and tumor grade in CCA could not be assessed due to small sample numbers, (Figure 3.6).

CCA exhibited a mean score of 9.9 (±2.2 SD) while HCC showed a mean score of 2.2-(±2.3 SD); this difference was significant ($p= 0.0001$).

Double immunostaining was performed to determine the co-localization of Shh and BMP4 proteins. Shh was mainly expressed in the subluminal epithelial cells of bile duct tumors (Figure 3.7). In contrast, BMP4 expression was exclusively located in the stromal cells of the bile duct tumors, as illustrated in (Figure 3.7).

Although Shh was detected in all three CCA cell lines, the expression of Shh was faint in two of the cell lines. Both HCC cell lines, HepG2 and HuH-7, exhibited a

band of strong intensity in comparison to CCA cell lines (Figure 3.8). The β-actin signal was present at comparable levels for cell samples loaded within the same gel.

3.4 Discussion

Hepatocellular carcinoma and cholangiocellular carcinoma are the most common primary liver malignancies of the human adult and have a poor prognosis. Despite the wide use of certain markers in detecting HCC and CCA, these two tumors are difficult to diagnose until they reach their advanced stages. The molecular mechanisms underlying both HCC and CCA development remain poorly understood. The hedgehog-signaling pathway fulfills an essential role during carcinogenesis by performing cell differentiation, proliferation and apoptosis; however, the role of Shh expression in liver tumors remains unclear. Previous studies have highlighted sonic hedgehog's pathological role in different types of malignant tumors (Ma *et al*., 2006; Morton *et al*., 2007; Yoshikawa *et al*., 2009). In the present study, the expression pattern of Shh was investigated in primary HCC and CCA liver tumors, using immunohistochemistry and Western blot analysis. Shh is frequently activated in liver carcinoma in this study. Although immunohistochemical analysis showed that Shh expression varies between liver carcinomas, Shh was strongly expressed in all CCA cases; however, slight to moderate Shh expression was detected in only 15 HCC cases. Moreover, the results demonstrate that Shh is strongly related to the tumor grade of HCC ($p = 0.016$). This is unclear for CCA tumors due to small sample numbers for G1 and G3 tumors. Interestingly, the strong expression of Shh was detected in both HCC

cell lines, HepG2 and HuH-7, using Western blot analysis. In contrast, the expression of the Shh protein in CCA varied from faint in OZ and HuH28 to moderate in HuCCT1.

Shh has undergone intensive investigation, and was described to have an essential role in the cell proliferation and differentiation during embryogenesis, as well as in tissue repair in adults (Omenetti and Diehl, 2008). Recently, a study by Pereira Tde *et al*. proposed that the upregulation of Shh performs an important function in tissue repairs during liver diseases, including liver inflammation (Pereira Tde, *et al*., 2010). In some tumors, including nevoid basal cell carcinoma syndrome, alterations in Ptch-1 and SMO genes activate the Hh pathway, promoting cell proliferation and carcinogenesis (Hahn *et al*., 1996; Reifenberger *et al*., 1998). However, overexpression of Shh was observed in some tumors, including colorectal carcinoma. Therefore, the literature has suggested that Shh fulfills a central role in carcinogenesis and tumor progression (Yoshikawa *et al*., 2009).

Several pathways were associated with the Hh pathway during both carcinogenesis and embryogenesis. A study by Marcelle C *et al*. showed that Shh might perform an antagonist action in the Wnt signaling pathway during the patterning of the dorsal somite (Marcelle *et al*., 1997). Moreover, the Wnt signaling pathway is associated with the Hh pathway during carcinogenesis. Specifically, it has been reported that Indian hedgehog is an antagonist for the Wnt signaling pathway in colon carcinoma (van den Brink *et al*., 2004). BMP4 is

another important pathway found to play a central role in dorsal-ventral of neural tube formation during embryogenesis (Liem *et al*., 1995). Recent studies suggested that in some carcinomas, including breast carcinomas, BMP4 has a bi-potential function; first, BMP4 acts as a tumor suppressor during the early stages of tumor development; second, BMP4 serves as a promoter for tumor cell migration/invasion, especially when the tumors become more advanced and aggressive (Alarmo *et al*., 2013). Chapter 2 describes the expression pattern of BMP4 in both primary liver malignancies, HCC and CCA. There was higher expression of BMP4 in CCA tumors than in HCC tumors (Johnston *et al*., 2012). In this current study, the co-localization between Shh and BMP4 was investigated. Shh induces the expression of BMP4 during the embryonic development of mice and amphibians (Sasaki *et al*., 2004; Ishizuya-Oka *et al*., 2006). Strong stromal BMP4 protein localization was detected with activated epithelial Shh in CCA tumor cases. This preliminary data supports the concept that activated Shh may induce BMP4 expression in CCA, which might promotion of epithelial mesenchymal transition (EMT) in liver malignancies (See section 1.1.3).

In conclusion, these preliminary results detected a significant overexpression of Shh in epithelial liver malignancies, where the expression was greater in CCA than HCC. The strong expression of stromal BMP4 may be regulated via the Shh signaling pathway. These results suggest that Shh may perform a crucial function in liver carcinogenesis and may be used as a diagnostic marker for distinguishing CCA from HCC tumors, thus also serving as a promising therapeutic target.

However, the relationship between Shh and BMP4 should undergo further investigation in order to illuminate the role of this relationship in primary liver malignancies and address the current study limitations related to sample size and double immunostaining.

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Table 3.1. Demographic characteristics of cholangiocellular carcinoma (CCA) and hepatocellular carcinoma (HCC) tumor cases

Diagnosis	G1	G2	G3
Cholangiocellular carcinoma N=19	1	15	3
Hepatocellular carcinoma N=21	7	6	8

G1, low grade or well differentiated; G2, intermediate grade; G3, high grade or poorly differentiated (see Table 1.1 for a description of the tumor grading system).

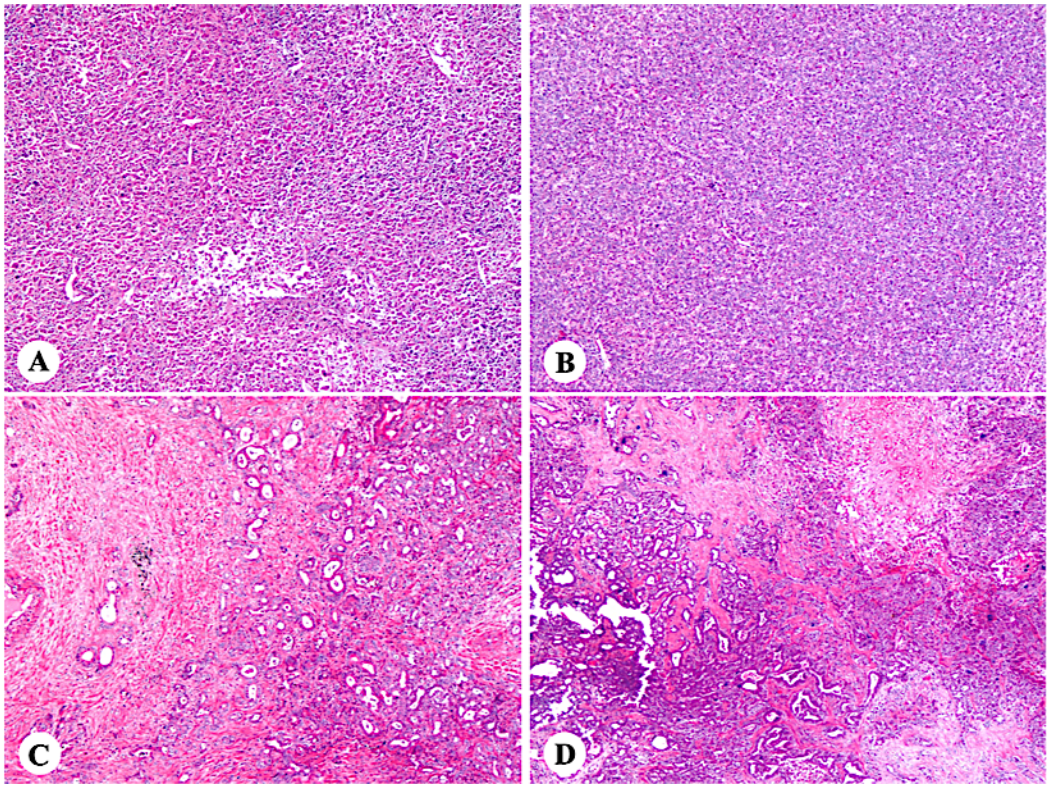

Figure 3.1. H & E satin for hepatocellular carcinoma (HCC) (A-B), and cholangiocellular carcinoma (C-D). A-D, 100X.

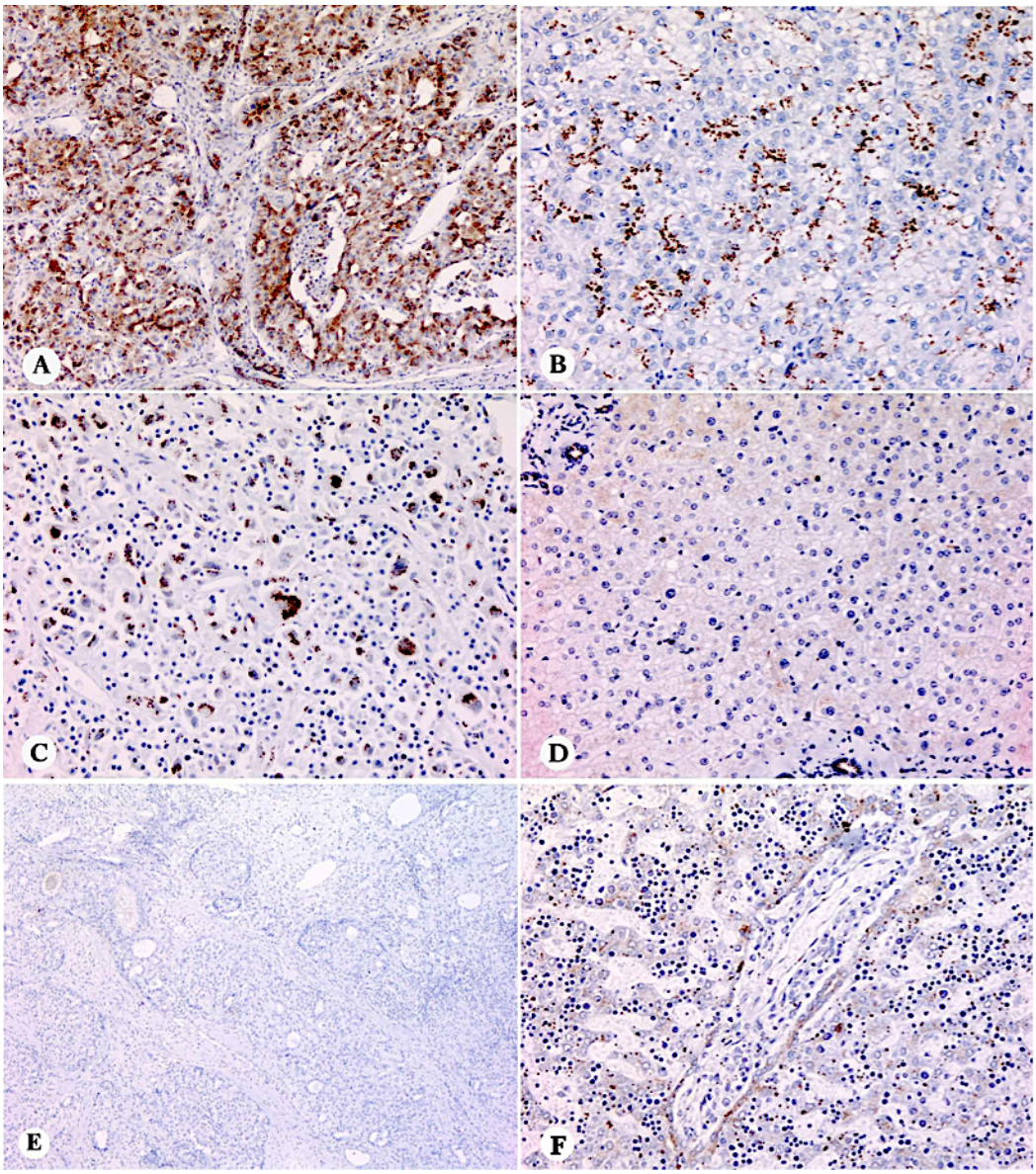

Figure 3.2. Immunolocalization of Sonic hedgehog (Shh) in hepatocellular carcinoma (HCC) (A-D). Strong (A) to slight or no expression (B-D) was seen in HCC. (E) Negative control. (F) Shh (brown staining) in normal fetal liver as a positive control (A, 100X; B, 200X; C, 200X; D, 200X; E, 100X; F, 100X).

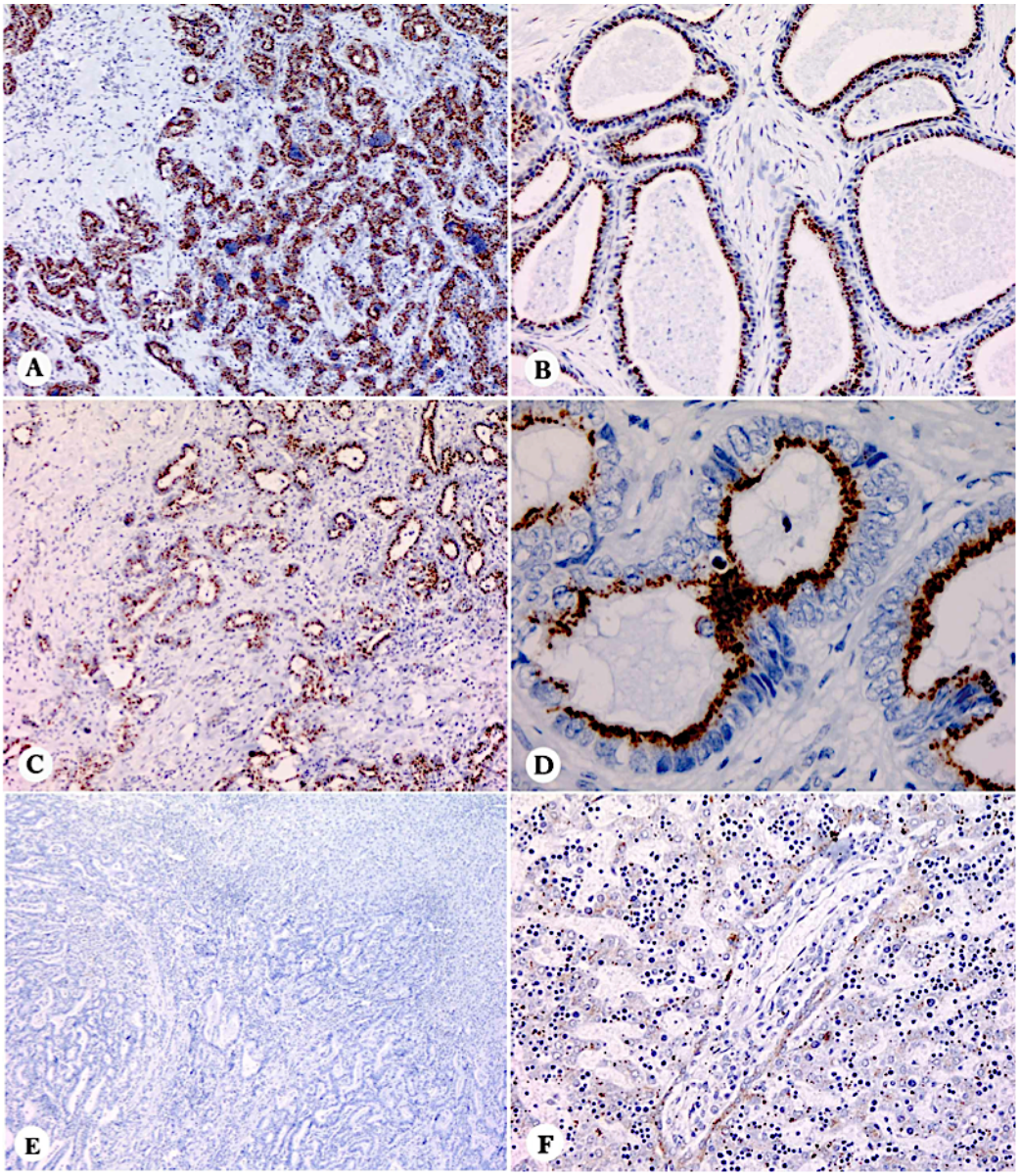

Figure 3.3. Immunolocalization of Sonic hedgehog (Shh) in Cholangiocellular carcinoma (CCA). Diffuse strong (A-D) expression of Shh was seen in the subluminal epithelial cells of the bile duct tumor (CCA). (E) Negative control. (F) Shh (brown staining) in normal fetal liver as a positive control (A, 100X; B, 200X; C, 200X; D, 200X; E, 100X; F, 100X).

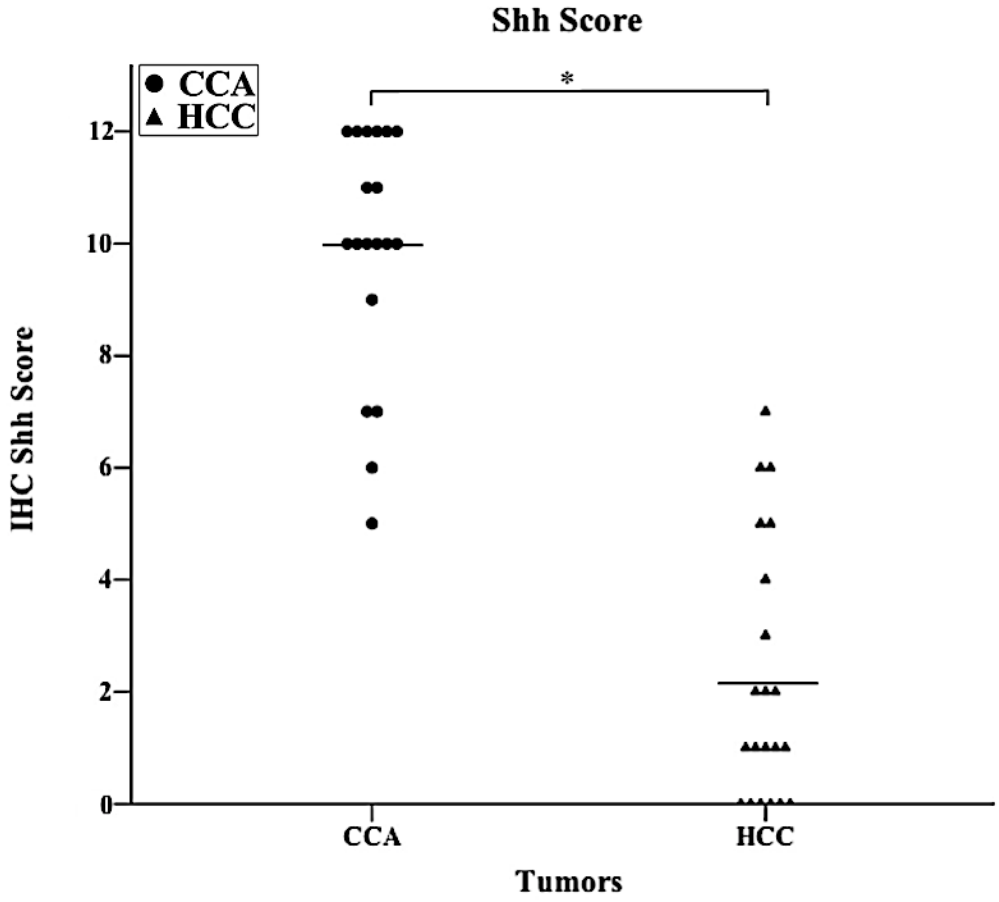

Figure 3.4. Sonic hedgehog (Shh) IHC score in cholangiocellular carcinoma (CCA) and hepatocellular carcinoma (HCC). p = 0.0001

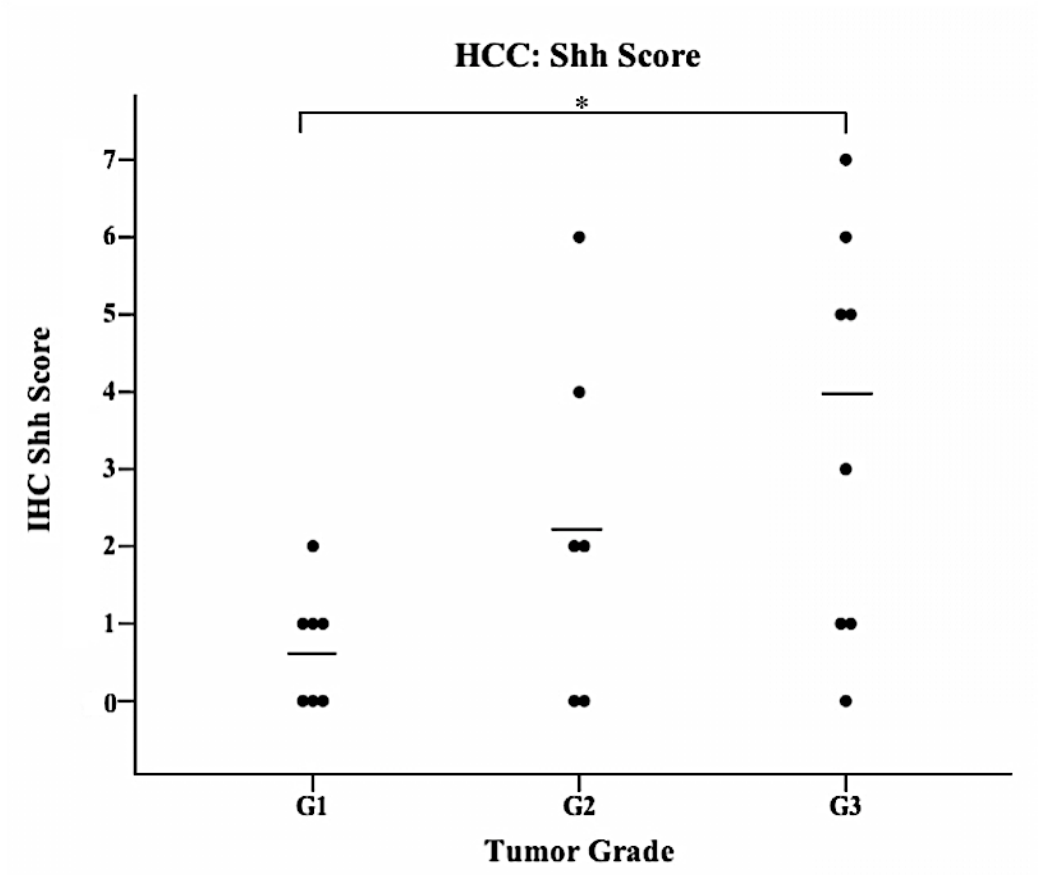

Figure 3.5. Sonic hedgehog (Shh) IHC score and tumor grade of hepatocellular carcinoma (HCC). p = 0.016

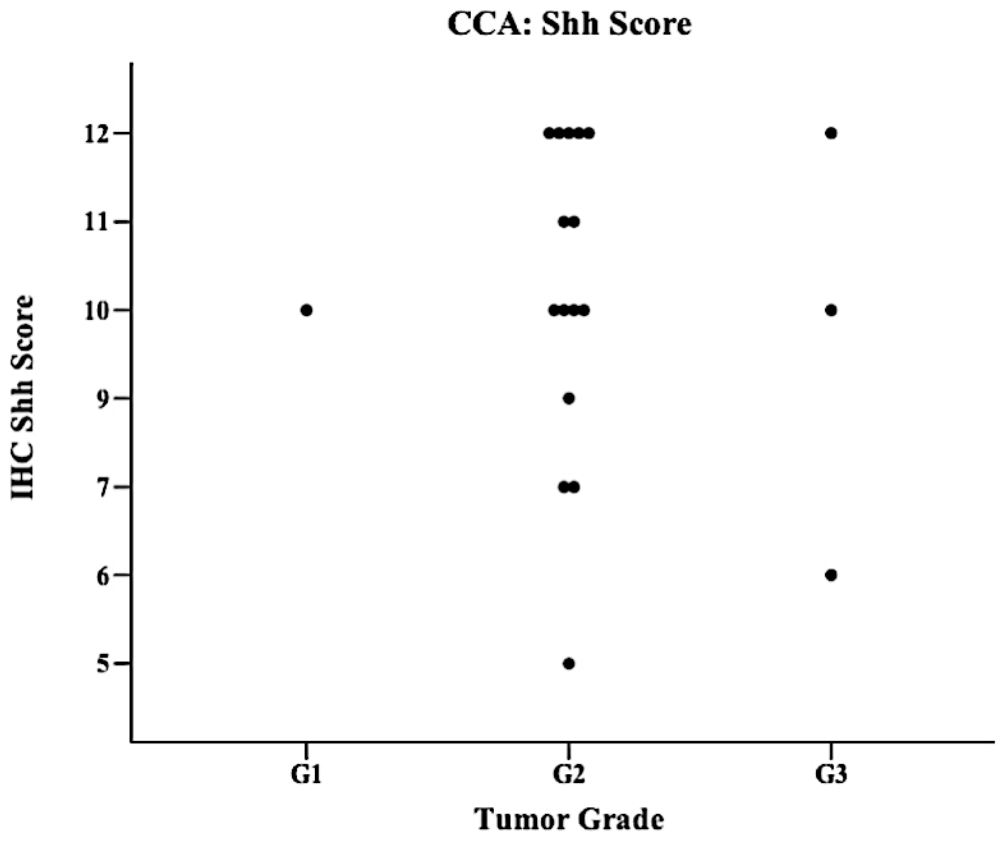

Figure 3.6. Sonic hedgehog (Shh) IHC score and tumor grade of cholangiocellular carcinoma (CCA).

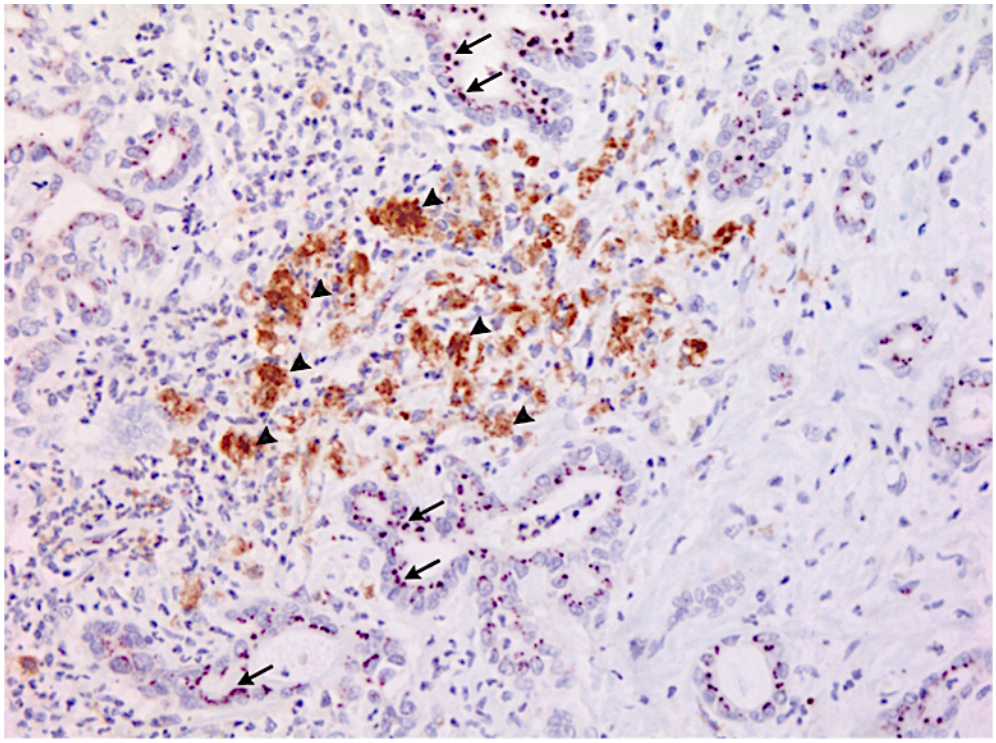

Figure 3.7. Double immunolocalization of Shh (purple) and BMP4 (brown) in cholangiocellular carcinoma (CCA). Strong epithelial cell expression of Shh (long arrows) at the subluminal region of bile duct tumors. Strong stromal cell expression of BMP4 (small arrows) in bile duct tumor. 200X

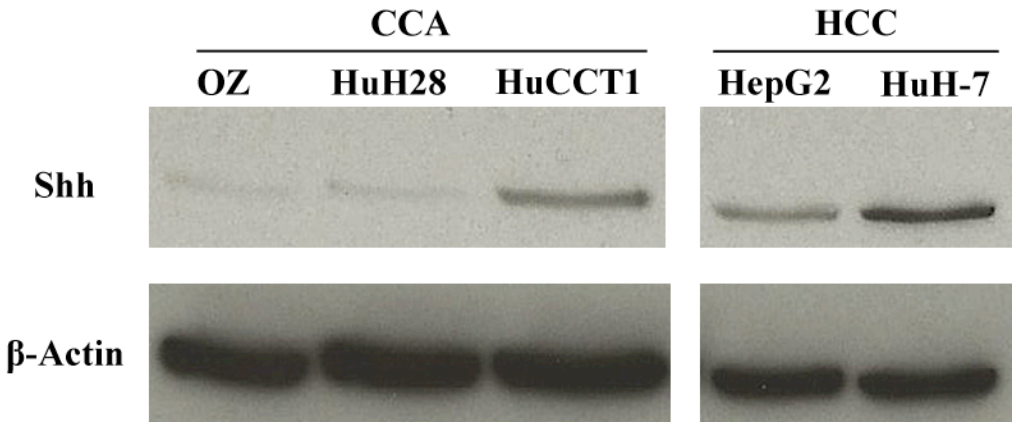

Figure 3.8. Western blot technique of Shh expression in two hepatocellular carcinoma cell lines (HepG2 and HuH-7), and three cholangiocarcinoma cell lines (OZ, HuH28, and HuCCT1). β-actin detection was used as loading control for constitutive protein expression to ensure consistent loading in each gel well.

Chapter 4: Discussion

4.1 General discussion

Liver neoplasms represent one of the most serious global diseases, with a high mortality rate. This condition, which results in more than 500,000 annual fatalities, possesses unclear etiological factors. Specifically, the molecular mechanisms underlying liver neoplasms remain poorly understood. The two common primary neoplasms of the liver involve hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCA); these tumors are usually detected at advanced phases of pathogenesis, accounting for their role as the silent killer. In order to understand the pathogenesis mechanisms of these carcinomas, several studies have investigated the molecular aspects of these neoplasms, including oncogenes such as k-ras and/or tumor suppressors such as p53 (Ahrendt *et al*., 2000; Hussain *et al*., 2007; Rizzi *et al*., 1996). Current studies attempt to provide new insight into liver cancer by investigating the role of the embryonic molecules in carcinogenesis and tumor progression, including molecular morphogens such as the hedgehog signaling pathway and the BMP signaling pathways. Accordingly, the aim of this study was to investigate the differential and co-localization expression of morphogen proteins, including Shh, BMP4 and TWSG1 and their relationship to tumor progression in both human HCC and CCA tumors and human cell lines.

4.2 Differential expression and localization patterns of TWSG1 and BMP4 proteins in HCC and CCA tumors, and cell lines

Chapter 2 of this study focused on the expression patterns and co-localization of BMP4 and its component TWSG1 proteins in both HCC and CCA tumors, and cell lines through immunohistochemical analysis and the Western blot technique. Furthermore, I investigated the relationship between ”morphogen proteins and tumor grade. Strong TWSG1 expression was located at the glandular, stromal and epithelial sites of liver neoplasms, which was twice as strong in CCA (83%) versus HCC (39%) tumors. This finding suggested that TWSG1 might perform a role in the glandular differentiation of liver neoplasm cells. Interestingly, I also found strong signals of TWSG1 in one of the CCA cell lines, OZ, which is metastatic. In conjunction, these findings suggest that TWSG1 might fulfill a function in cell differentiation remodeling as well as increasing the tumor progression and invasiveness of liver carcinomas.

BMP4 is a member of the TGF-β superfamily, which is reported to have bi-potential action in cellular apoptosis (Shirai *et al*., 2011) and migration (Guo *et al*., 2012; Lorente-Trigos *et al*., 2010) in some cancers. In this study, BMP4 was strongly expressed in all CCA tumor but not HCC tumors; only one case demonstrated the positive expression of BMP4 in HCC tumor. In contrast, BMP4 expression was present in both HCC and CCA cell lines; however, the expression was extreme in one of the HCC cell lines, well-differentiated (G1) HuH-7 (See section 2.2.4), suggesting that BMP4 may act as a tumor suppressor during the

early stage of liver carcinogenesis (Shirai *et al*., 2011; Deng *et al*., 2007) and then later a tumor promoter resulting in poorly-differentiated tumor cells (Katoh and Terada, 1996). In addition, TWSG1 is known as an antagonist and agonist of BMPs, including BMP4 (Chang *et al*., 2001; Oelgeschlager *et al*., 2000). The co-localization of BMP4 and TWSG1 proteins was undetectable in the microscopy optical field. Immunohistostaining reveals strong expression of BMP4 present at the stromal cells of CCA and HCC tumors, whereas TWSG1 expression was present in both stromal and epithelial cells of HCC and CCA tumors (Figure 2.13).

4.3 Differential expression patterns of Shh protein and its co-localization with BMP4 in HCC and CCA tumors, and cell lines

BMP4 and Shh are involved in cellular proliferation and differentiation, where these two proteins interact with each other during embryonic development (Merchant, 2012), as well as during carcinogenesis (Hahn *et al*., 1996;Reifenberger *et al*., 1998) (See section 1.3). In Chapter 3, I investigated the differential expression of Shh and its co-localization with BMP4 in HCC and CCA; this investigation was performed using immunohistochemistry and Western blot techniques. Secondly, I investigated the correlation between Shh protein expression and tumor grades in both HCC and CCA human tumors and human cell lines. Shh expression was 5 times greater in CCA tumors than HCC tumors. Moreover, there was a significant correlation between Shh expression and tumor grades of HCC tumors but not CCA tumors, due to small sample numbers for G1 and G3 grade CCA tumors. The expression of Shh protein increased from well-

differentiated to poorly-differentiated tumors. Interestingly, the expression pattern of the Shh protein was stronger in HCC cell lines than in those of CCA; however, one CCA cell line, a moderately-differentiated (G2) HuCCT1 (See section 2.2.4), did have strong Shh expression. Although BMP4 was expressed, I found no significant co-localization between Shh and the BMP4 protein. Interestingly, the stromal expression of BMP4 protein may be induced by the epithelial expression of Shh protein (Wang *et al*., 2010), which might promote the epithelial mesenchymal transition (EMT) in liver malignancies (Figure 3.7) (Wang *et al*., 2010). Further studies in this aspect are needed to demonstrate the interaction between Shh and BMP4 protein.

4.4 Limitations and future studies

The various studies in this thesis each possess their own set of limitations and possible future studies. In Chapters 2 and 3, a limited number of patient samples were used. For instance, in Chapter 2, 13 cases of HCC and 12 cases of CCA were studied for TWSG1, while only 5 cases of HCC and 8 cases of CCA were probed for BMP4. Furthermore, in Chapter 3, 21 cases of HCC and 19 cases of CCA were investigated for Shh. This limitation hindered the statistical analysis in some categories. Specifically, a limitation exists in the small number of cases and their tumor grades, complicating the efforts to perform a correlational analysis of IHC markers and tumor grades. Accordingly, future studies must increase the number of HCC and CCA tumor samples for each tumor grade. Moreover, in Chapter 3, patient histories were not available during this study to investigate whether Shh protein is a prognostic marker or not. Hence, subsequent research can investigate

the correlation of Shh expression in treated versus non-treated patients. Additionally, double immunostaining was an inexpensive approach performed to observe the co-localization of TWSG1 and Shh with BMP4 protein, but this was not an ideal technique. Specifically, the aim of the co-localization involves observing the spatial overlap of two or more different stains. Accordingly, future studies can investigate co-localization using immunofluorescent staining and visualized by confocal microscopy to obtain more details of the co-localization between Shh and BMP4 protein, and TWSG1 and BMP4 protein.

4.5 General conclusion

This thesis provided insight into a possible interaction between morphogen proteins, including Shh and BMP4 in solid tumors of HCC and CCA. This possible interaction may be associated with the HCC and CCA tumor progression. To the best of my knowledge, this study represents for the first time that TWSG1 protein has been attributed to poor tumor grade in human CCA and HCC, and may be used as a tumor marker to differentiate CCA from HCC. Furthermore, there was no correlation between BMP4 and its component TWSG1 expression in CCA and HCC in this study, suggesting that BMP4 and TWSG1 may be activated independently during liver carcinogenesis, however, this is only an speculation and further studies are needed to confirm this idea. Most importantly, in the second study, I identified the fact that Shh might be useful as a tumor marker to differentiate CCA from HCC. Moreover, I also identified strong stromal BMP4 expression in CCA and proposed that it may be induced by epithelial Shh

promoting EMT during liver carcinogenesis, although further investigations are needed to confirm this aspect.

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