

Growth of hepatocellular carcinoma and liver regeneration

by

Ji-Hua Shi



Department of Transplantation Medicine & Institute for Surgical Research,
Oslo University Hospital & Faculty of Medicine, University of Oslo

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ABBREVIATIONS

AJCC/UICC	<i>American Joint Committee on Cancer/Union internationale contre le cancer staging system</i>	HCV	<i>Hepatitis C virus</i>
		HGF	<i>Hepatocyte growth factor</i>
		HPC	<i>Hepatic progenitor cell</i>
αFP	<i>Alpha fetoprotein</i>	HSC	<i>Hepatic stellate cells</i>
CAPNS1	<i>Calpain-small subunit 1</i>	IGFBP1	<i>Insulin growth factor binding protein-1</i>
CLIP	<i>The Cancer of the Liver Italian Program</i>	JIS	<i>The Japanese Integrated Staging</i>
PST	<i>WHO performance status test</i>	IL-6	<i>Interleukin- 6</i>
CTP	<i>Child-Turcotte-Pugh score</i>	LDLT	<i>Living-donor liver transplantation</i>
EC	<i>Endothelial cells</i>		
EGF	<i>Epidermal growth factor</i>	MAPK	<i>Mitogen-activated protein kinase</i>
EMT	<i>Epithelial to mesenchymal transition</i>	MMP	<i>Matrix metalloproteinase</i>
ECM	<i>Extracellular matrix</i>	mTOR	<i>Mammalian target of rapamycin</i>
GH	<i>Growth hormone</i>	NO	<i>Nitric oxide</i>
HBV	<i>Hepatitis B virus</i>	OLT	<i>Orthotopic liver transplantation</i>
HCC	<i>Hepatocellular carcinoma</i>	PEI	<i>Percutaneous ethanol injection</i>
PDGF	<i>Plate differentiation growth factor</i>	TGF-α	<i>Transforming growth factor alpha</i>
PGs	<i>Prostaglandins</i>	TGF-β	<i>Transforming growth factor beta</i>
PI3K	<i>Phosphatidylinositol-3 kinase</i>	TNF-α	<i>Tumor necrosis factor alpha</i>
PVL	<i>Portal vein ligation</i>	TNM	<i>Tumor-Node-Metastasis staging system</i>
TACE	<i>Transcatheter arterial chemoembolization</i>	VEGF	<i>Vascular endothelial growth factor</i>

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numbers.

I.

Shi JH, Huitfeldt HS, Suo ZH, Line PD. Growth of hepatocellular carcinoma in the regenerating liver. *Liver Transpl.* 2011 Jul; 17(7): 866-74.

II.

Shi JH, Scholz H, Henrik S. Huitfeldt, Line PD. The effect of hepatic progenitor cell on experimental hepatocellular carcinoma in the regenerating liver. *Scandinavian Journal of Gastroenterology*, in press, 2013

III.

Shi JH, Liu SZ, Wierød L, Scholz H, Anmarkrud JA, Henrik S. Huitfeldt, Zhang SJ, Line PD. RAF-targeted therapy for hepatocellular carcinoma in the regenerating liver. *Journal of Surgical Oncology.* 2013; 107: 393–401

1. INTRODUCTION

1.1 Hepatocellular carcinoma

1.1.1 Epidemiology, diagnosis and treatment modalities

Hepatocellular carcinoma (HCC) ranks sixth in frequency worldwide among all malignancies with more than 500 000 new cases diagnosed annually, and is the third leading cause of cancer mortality with a mortality-to-incidence ratio exceeding 0.9 (1-3). HCC is the most common primary malignancy of the liver accounting for 80%-90% of primary malignant liver tumors, and is currently the leading cause of death amongst cirrhotic patients (2, 4-6).

Well-defined **risk factors** for hepatic carcinogenesis have been identified (7, 8). The majority of HCC (95% in the western, 60% in Asian countries) develops in the context of liver cirrhosis, and this pre-neoplastic condition is the strongest predisposing factor (8, 9). More than 80% of HCC arise in patients with chronic liver disease due to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection(5, 6, 10, 11). Cirrhosis from non-viral causes such as alcoholism, hereditary hemochromatosis and primary biliary cirrhosis are also associated with an elevated risk of HCC. Furthermore, concomitant risk factors such as HBV, HCV infection in addition to alcoholism, tobacco use, diabetes or obesity increase the relative risk of HCC development, as demonstrated in numerous human studies and further supported by animal models (12-16). The **incidence** of HCC varies worldwide by geographic area from a relatively rare, like in North America and Europe, to a comparatively common as in Southeast Asia and sub-Saharan Africa (Table 1). Southeast Asia and sub-Saharan Africa have an incidence rate of HCC that ranges from 150 to 500 per 100 000 population, primarily because of the endemic nature of HBV in those regions (14, 16, 17). Chronic HBV carriers have a 100-fold relative risk for developing HCC, with an annual incidence rate of 2–6% in cirrhotic patients (18). In contrast, the incidence of HCC in developed countries is low but increasing (19, 20). The increasing burden of chronic viral hepatitis, fatty liver disease and alcohol-related liver disease may account for the observed increment in HCC incidence (21). In North America, Europe and Japan, HCC develops in cirrhotic livers mostly due to hepatitis C or alcohol abuse (22). Approximately 20–30% of the estimated

170 million HCV-infected individuals worldwide will develop cirrhosis (23). Once cirrhosis is established, the annual incidence of HCC is about 3–5%, and one third of the patients will develop HCC during their lifetime (22, 24, 25).

Table 1. Incidence of Hepatocellular carcinoma per 100 000 inhabitants worldwide (1)

Geographical area	Age-adjusted incidence (M/F)	Leading risk factors
Asia and Africa		
East Asia	35.4/12.6	
Middle Africa	24.2/12.9	
Southeast Asia	18.3/5.7	
America		
Southern	4.8/3.6	
Northern	4.1/1.6	
Europe		
Southern	9.8/3.4	
Western	5.8/1.6	
Northern	2.6/1.3	
Developing countries	17.4/6.7	HBV cirrhosis
Developed countries	8.7/2.8	HCV cirrhosis Alcoholism cirrhosis
World	14.9/5.5	

The macroscopic features of HCC vary depending on the size of the tumor and the presence or absence of liver cirrhosis. **Gross morphology** at diagnosis may be divided into nodular (60%), massive (30%) and diffuse types (26). The nodular type is the most common, and is usually well demarcated and surrounded by a fibrous capsule within a cirrhotic liver. Multiple nodules may be scattered among the cirrhotic nodules with some dominant tumor nodule. The massive type consists of a large single mass with or without satellite nodules and is more often seen in a non-cirrhotic liver. The diffuse type is relatively uncommon and consists of innumerable indistinct

small nodules scattered throughout a cirrhotic liver (27). The presence of a capsule, multicentricity and intravascular and biliary tract invasion are important gross features associated with dismal prognosis and short long-term survival (28-31).

Molecular pathology and genomic analysis have provided a better understanding of the mechanisms of hepatic carcinogenesis, cancer progression and have helped to identify new molecular targets that are linked to the prognosis of HCC. P53 and β -catenin mutations represent the two main genetic alterations in HCC (32, 33). Many cell signaling pathways are implicated in HCC pathogenesis, including the RAF/MEK/ERK pathway, phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, WNT/ β -catenin pathway, hepatocyte growth factor (HGF)/c-MET pathway and growth factor-regulated angiogenic signaling. Mutations in genes involved in cellular signaling have been identified, which could support the development of possible targeted therapies (32, 34). Important signaling pathways and genes linked to hepatic carcinogenesis and HCC cell biology are summarized in Table 2 and Table 3.

Table 2. Molecule-mediated signaling pathways in HCC pathogenesis (34)

Signaling pathways		Cell survival	Cell proliferation	Cell differentiation	Cell migration	Angiogenesis
Tyrosine kinase signaling pathways	HGF/c-MET	✓	✓		✓	✓
	EGF(TGF- α)/EGFR	✓	✓			
	IGF/IGFR	✓	✓		✓	
	VEGF/VEGFR					✓
	PDGF/PDGFR					✓
	TGF- β 1/SMAD			✓	✓	
Intercellular kinase signaling pathways	RAF/MEK/ERK	✓	✓	✓		✓
	PI3K/AKT/mTOR	✓	✓			✓
	Wnt/ β -catenin	✓	✓	✓		✓
	Hedgehog		✓	✓	✓	
	NK- κ B, JAK/STAT3	✓	✓	✓	✓	✓

HGF, hepatocyte growth factor; EGF, epidermal growth factor; TGF- α , transforming growth factor alpha; IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor; PDGF, plate differentiation growth factor; TGF- β , transforming growth factor beta

Table 3. Dysregulation of critical genes and gene products in HCC (32)

Cell function	Gene	Expression
Proliferation and differentiation	β -catenin	Increased
	E-cadherin	Decreased
	c-myc	Increased
Growth factor signaling	TGF- α	Increased
	PTEN	Decreased
	IGF-1	Increased
	IGF-2(M6PR)	Decreased
	IGFBP-3	Increased
	PDGFRA	Decreased
	HGFR	Increased
	HGF	Increased
	EGFR	Increased
	TGF- β 1	Decreased
	Smad2	Decreased
Cell cycling	P53	Decreased
	P16	Decreased
	P14	Decreased
	P27kip	Decreased
	CyclinD1	Decreased
	Rb	Increased
	Survivin	Increased
Angiogenesis	VEGF	Increased
	VEGFR-2	Increased
	Angiopoietin-2	Increased
Metastasis	Rho C	Increased
	Kangai 1	Decreased
	MMP-9	Increased
	MMP-14	Increased
	Topoisomerase 2A	Increased
	Osteopontin	Increased

Standard **diagnostic studies** include ultrasonography with or without contrast, computed tomography (CT) scanning, magnetic resonance imaging (MRI), ultrasound-guided needle core biopsy or fine-needle aspiration biopsy in selected cases, and determination of serum alpha fetoprotein (AFP) (35). Imaging modalities are essential for identification and localization of HCC tumors, and are used in the staging of patients. AFP level are elevated in about 70% of HCC cases, usually above 400 ng/mL (normal 20-40 ng/mL). When AFP is significantly elevated and a tumor is observed in the liver, it is reasonable to assume a diagnosis of HCC. The diagnostic accuracy is dependent on a number of variables: tumor size, presence of cirrhosis and expertise and experience of the imaging operator and the interpreter. Liver biopsy might be indicated when diagnosis based on morphological investigations alone is unclear, but is usually not required. Based on the structural organization of tumor cells, World Health Organization has suggested that HCC should be classified into the following **histological types**: trabecular/sinusoidal type, pseudoglandular/acinar type, and compact/scirrhous sclerosing agent type. The tumor can also be graded based on the degree of cell differentiation into well, moderately, poorly differentiated and undifferentiated (36).

The **prognosis** without specific treatment is poor with a median survival of early and advanced HCC being 6-9 months and 1-2 months respectively (37). The asymptomatic nature of early HCC, lack of awareness and poorly defined screening strategies, leads to that approximately 80% of patients present with advanced or unresectable disease at the time of diagnosis and have a very poor prognosis (38). Hence, the 5-year population based survival rate for HCC patients at large is only 7%. In some developed countries, 30–40% of patients are now being diagnosed at earlier stages where potential curative treatment modalities can be offered (39). Assessments of HCC outcome and planning of therapeutic strategy for HCC patients need to consider the tumor stage, the severity of the underlying liver disease and liver function. Thus, the various classification and staging systems for HCC are more complex than for other cancers, because the prognosis depends not only on the tumor status but also on the underlying liver disease. The most widely accepted **staging systems** for predicting the survival of patients is The American Joint Committee on Cancer/Union internationale contre le cancer Tumor-Node-Metastasis staging system (AJCC/UICC TNM staging system, 7th edition) (Table 4) (5, 6), Okuda

score and The Japan Integrated Staging (JIS) score (Table 5) (40), The Cancer of the Liver Italian Program (CLIP) score (41) and The Barcelona Clinic Liver Cancer (BCLC) staging system(42, 43).

Table 4. Summary of HCC classification from AJCC/UICC cancer staging* (5, 6, 44)

Group	Subgroup	Description
T1		Solitary tumor without vascular invasion
T2		Single tumor with vascular invasion, or multiple tumors, none >5cm
T3	a	Multiple tumors, any >5cm
	b	Tumors involving a major branch of portal or hepatic vein
	c	Invasion of adjacent organs other than gallbladder
T4	a	Regional lymph node metastasis
	b	Distant metastasis

* Prognosis value of Ishak fibrosis stage is also recommended.

Table 5. The Japan Integrated Staging (JIS) score for Hepatocellular Carcinoma (40)

Variable	Score			
	0	1	2	3
Child-Turcotte-Pugh (CTP) class	A	B	C	-
Tumor-Node-Metastasis (TNM) stage by Liver Cancer Study Group of Japan	I	II	III	IV

JIS score (0-5): Sum score of CTP class and TNM stage.

There is no consensus as to which staging system is best in predicting the survival of patients with HCC (45, 46). In general, pathologic staging systems such as the AJCC/UICC predicts prognosis better than clinical systems do, particularly when assessing the outcomes after liver resection. Okuda, CLIP and BCLC are more useful for predicting outcomes in patients with poor liver function who have advanced HCC and are undergoing nonsurgical therapy (47). JIS score, combining Child-Turcotte-Pugh (CTP) class and the modified TNM stage with consideration of

the patients' clinical condition, has been shown to be superior to the CLIP scoring system (40, 48). The BCLC staging system involves tumor-related parameters, patients' clinical condition (WHO Performance Status) and liver function (CTP class). BCLC links the stage of disease directly to respective treatment strategies and was recently updated (Figure 1). Based on BCLC scoring, HCC disease can be categorized as very early, early, intermediate, advanced and terminal stage.

The consensus of the American Hepato Pancreato Biliary Association (2010) recommended the use of AJCC/UICC staging system to predict outcome following resection or liver transplantation, and the BCLC staging system for patients with advanced HCC who are not candidates for surgery(49).

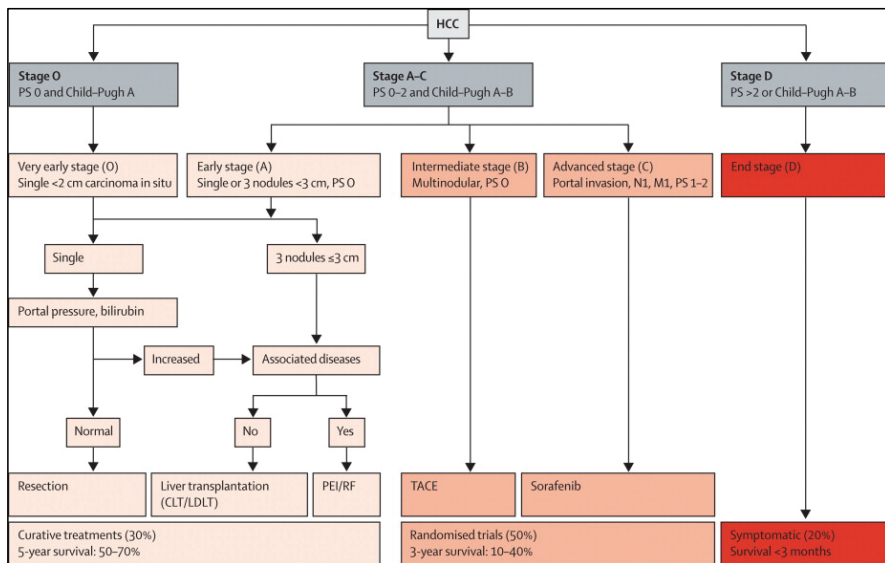


Figure 1. Barcelona-Clinic Liver Cancer staging classification and treatment schedule (45, 46)

CTP, Child-Turcotte-Pugh; OLT, orthotopic liver transplantation; PEI, percutaneous ethanol injection; PST, WHO performance status test; RFA, radiofrequency ablation; TNM, Tumor-Node-Metastasis.

Since HCC is causally related to chronic viral hepatitis (HBV or HCV) and various chronic liver diseases, prevention of these causal liver diseases, particularly viral hepatitis, could lead to **prevention** of HCC. Prophylaxis against mother-to-child transmission of HBV and prevention of HCV transfer associated with medical interventions, for example, blood transfusion, is strongly recommended as a secondary preventive measure against the development of chronic viral hepatitis and HCC. HBV vaccination was launched in Taiwan in 1984 and led to a substantial reduction in the prevalence of HBsAg positivity from 10% to 1%, resulting in a decreased incidence of HCC in children within 10-year of observation (50, 51). In Singapore, short- and long-term measures for the prevention of HCC have been tried, including introduction of prophylactic HBV immunization, discouragement of alcohol and tobacco consumption, screening for aflatoxin in food materials, and testing for HCV. Prophylactic administration of lymphoblastoid interferon-alpha has also been tried in high-risk patients with favorable results. An important reason for the high mortality rate in patients with HCC is the lack of effective **treatment options**, especially for those with advanced disease. Treatments for HCC can be divided into curative and palliative regimens. Curative treatments, such as liver resection, liver transplantation, and local ablation, may induce complete responses in a high proportion of patients and thereby improve survival. Well documented palliative treatments include transcatheter arterial chemoembolization (TACE) and the tyrosine kinase inhibitor Sorafenib (52). Other measures like intra-arterial or systemic chemotherapy (5-fluorouracil, doxorubicin, interferon alpha, cisplatin, oxaliplatin, 5-fluorouracil/leucovorin, etc)(53), radiotherapy, immunotherapy or hormonal therapy have also been tried in order to improve survival. In general, the paucity of effective and well-tolerated treatments for advanced HCC highlights the need for new therapeutic approaches (1, 54, 55).

1.1.2 Curative treatment modalities for HCC

The only potential curative treatment options are partial hepatectomy, liver transplantation and local ablation. These three modalities have their separate indications, although some overlapping indication groups might exist. They have not been compared in randomized

controlled trials, partly due to the need for very large sample sizes, and complex trial design requirements (1).

Liver resection may be performed in patients with adequate liver function, anatomically manageable location of disease, no extrahepatic metastases and absence of severe portal hypertension. Technical improvements and better patient selection have contributed to an overall 5-year survival rate of 40-70% after resection and a significant decrease in operative and postoperative mortality, which is now less than 5% (56-59). The safe limits for the extent and type of liver resection are based on assessment of hepatic function. The Child-Turcotte-Pugh grading continues to be the most useful clinical evaluation system. Indocyanine green (ICG) clearance test is widely used to identify good candidates for liver resection in Asia (60), and portal pressure or the model for end-stage liver disease (MELD) (61) score is used in Europe (62). The best indications for resection is small and single-nodule HCC with normal liver function (Child-Pugh class A), which means normal bilirubin, no sign of portal hypertension (absence of ascites), ICG clearance at 15 min less than 10% or MELD ≤ 8 . This might yield a 5-year survival rate of approximately 70% (1, 63). However, only 20% to 30% of patients with HCC are eligible for resection due to advanced or multifocal disease or inadequate functional hepatic reserve (64). Larger HCC may be resected with good results when the tumor is well-differentiated and slow-growing (56).

Since 1996, **orthotopic liver transplantation (OLT)** has been considered the optimal treatment for small HCC in cirrhosis since it cures the tumor, the cirrhosis and removes preneoplastic lesions at the same time. This was demonstrated by Mazzaferro and coworkers in a seminal paper reporting an overall 4-year survival rate of 85% and relapse-free survival of 92% (65-67). Retrospective studies indicate a better overall and relapse-free survival after OLT in selected patients, compared with resection or local ablation (57, 67, 68). Classically, OLT is indicated in patients with cirrhosis and a single HCC lesion with a diameter of ≤ 5 cm or with three nodules of ≤ 3 cm (Milan criteria) (57). Due to the rising demand for effective treatment options and increasing incidence of HCC, the criteria for OLT have been extended to include larger HCC tumors at various centers (66, 69). Although Transplantation is considered the best treatment for early stage HCC in cirrhotic patients, it is feasible in fewer than 5% (63).

About 50% of HCC patients who were initially candidates for OLT will become ineligible for OLT if the median waiting period exceeds 1 year (70, 71). In view of these problems, **living-donor liver transplantation (LDLT)** is increasingly discussed as an additional option (72). This enables avoidance of long waiting time before transplantation, increases the number of available liver grafts and is therefore an effective approach to reduce the dropout rate. The following arguments support the concept of LDLT in patients with HCC and cirrhosis: better clinical condition of the patient at the time of transplantation due to a scheduled procedure; better graft function because of a healthy graft, optimal organ harvest, conservation, and reduced cold ischemia time; a significantly reduced waiting time, resulting in a reduced risk of tumor progression and a potentially better long-term survival. Primary graft failure and the risk to the donor including donor deaths and other complications present ethical concerns that cannot be disregarded. The complication rate in LDLT donors in Japan, North America and Europe ranges between 9.2% and 40%, and the mortality risk have been reported to range from 0% to 0.61% (73-76).

Both transplantation and resection are limited in their applicability, and a significant proportion of the patients are offered **local ablative therapies** that can compete in efficiency with surgical resection of small HCC nodules (64). Moreover, local ablation can be applied to a larger number of patients with multifocal presentation and comorbidities that might contraindicate liver surgery (77, 78).

Radiofrequency ablation (RFA) (79) has become the most frequently used form of ablative therapy. RFA is designed to destroy tumor tissue with ionic agitation and frictional heating (80, 81). The main advantage of RFA is the ability to treat a variable number of liver lesions, and to increase resectability rate by combining RFA with resection if several lesions throughout the parenchyma have to be removed simultaneously (82). In addition, this is not offset by an increase in morbidity and high rate of complications (83). RFA achieves a local complete response rate of more than 80% (84), improves the overall survival compared with other modalities such as chemotherapy or percutaneous ethanol injection (85) (86), and has even shown a similar 5-year survival rate as a primary liver resection in selected series (87). Due to thermal diffusion in the tissue, there is a limit with regards to how large tissue volumes that can

be ablated effectively. General consensus guidelines from North America and Japan recommend that RFA can be used for three or fewer HCCs with a diameter of ≤ 3 cm (86, 88).

Local ablation techniques as well as liver resections are more frequently followed by tumor recurrence than liver transplantation. Hence, there is a need for a better understanding of biological mechanisms promoting tumor recurrence and thereby gain insight how progression of HCC can be reduced or halted (89).

1.1.3 Tumor recurrence after liver surgery: risk factors, prevention and treatment

Although resection and local ablation can achieve long-term control in a high proportion of patients with early HCC, recurrence rates are high (approximately 50% at 3 years) despite of successful surgery achieving R0 resection or complete regression (90). The observed frequency of tumor recurrence following resection in patients with advanced HCC depends on the duration of the follow-up (91). In various series, 43- 65% of the patients displayed recurrences within 2 years of removal of the first tumor (92-94), and this have been shown to be as high as 85 % after five years of observation in some studies (16, 95-97). Although OLT offers a potential cure for selected patients with HCC, tumor recurrence is still a major cause of mortality in transplanted patients, even when selected within Milan or UCSF criteria (66, 69). Some retrospective studies have suggested a higher recurrence rate after LDLT compared with conventional OLT (98, 99). For RFA, a similar high rate of HCC recurrence as seen in resected patients have been reported, and this could have an adverse effect on patient survival in patients treated with RFA alone (100, 101)

The liver is the primary site of recurrence in 30-91% of patients (93, 102-107). Microvascular invasion, poor histological differentiation, and satellite lesions predict a bad prognosis (57, 108). Recurrence after surgical therapy might be divided into **two categories**: early recurrences appear in the first 2 years after treatment, and is considered to be caused by dissemination of residual hepatic tumors due to micrometastases or a multicentric tumor in the liver remnant missed by preoperative staging or incomplete resection surgery (57, 102, 109). Late recurrences (so-called *de novo* cancer) occur more than 2 years after surgery, and is most likely a result of the underlying carcinogenic liver disease or virus (85, 110, 111). Thus, intrahepatic recurrence

may arise through two different mechanisms: growth of residual tumor cells or a metachronous, multicentric carcinogenesis. Intrahepatic residual tumor appears to be more frequent than repeated "de-novo" tumors (102, 112).

This distinction may have prognostic and therapeutic implications. Patients who are presenting with a solitary recurrence late after initial therapy and good liver function classified as Child grade A, would appear to be good candidates for re-resection, as opposed to patients with early recurrent hepatic tumors, which are often multifocal (112).

Numerous risk factors related to tumor characteristics at the time of the primary treatment are influencing the hepatic recurrence rate (91, 94, 97, 108, 113). A tumor size larger than 5 cm (66, 103, 113), multiple nodules (103, 113-115), the presence of satellite nodules, vascular involvement (94, 97, 102, 103, 108, 116), the absence of capsule formation (103, 108, 117, 118), preoperative AFP level (95), DNA aneuploid content (94, 113), and other risk factors related to the patient and the underlying liver status, including cirrhosis, active hepatitis, and alcohol abuse (94, 118) are all well known risk factors. In addition a number of **surgical risk factors** include: free resection margin of less than 1 cm (93, 95, 107, 113, 116), perioperative transfusion (119, 120), and immunosuppression (121, 122). Recently some studies have suggested that the cellular and molecular changes induced by hepatectomy, including surgical stress responses and ischemia reperfusion injury may influence the kinetics of tumor growth and thereby contribute to recurrence (123-126).

The recurrence rate may be reduced by some pre- and post-operative **preventive measures** (91): avoiding manipulation of tumors, using an anterior approach to minimize the risk of tumor cell dissemination due to mobilization and manipulation of the liver (127), minimizing operative blood loss in order to avoid blood transfusion (128). The use of systemic chemotherapy after curative resection for HCC has been associated with a worse outcome (129). Some older investigations have proposed that postoperative regional chemotherapy or lipiodolisation may be an effective therapy to reduce recurrence and improve survival (130-132). Injection of polyphenolic acid has been reported to reduce the incidence of second primary HCC after resection (133). Recently the use of Interferon beta has been reported to be effective in preventing recurrence of HCC after complete resection or ethanol ablation in patients with HCV-

related chronic liver disease(134). Other investigations have reported promising results with the use of anti-angiogenic drugs on recurrence of HCC (135, 136). None of these methods have, however, gained widespread use, and need further validation in randomized controlled trials. Although recurrence following curative treatments is associated with a poor outcome in most cases, there is some evidence that selected patients with liver only recurrence might benefit from more aggressive approaches (137, 138). Consequently, all the **treatment modalities** in use for primary HCC, i.e. surgical resection and liver transplantation, local ablation, and TACE have been tried in this setting (16, 91-97, 139). Repeat hepatectomy appears to be the best treatment e with a reported resectability rate ranging from 10 to 77%. Patients treated by repeat hepatectomy have been reported to have better survival rates than those treated by other palliative methods (92, 104, 105, 114, 140). A longer interval from hepatectomy to recurrence is associated with improved survival (92, 104). TACE appears to be the best option in recurrent tumors that are unresectable due to multinodularity or inadequate functional liver reserve (91). TACE might in a few cases downstage the tumor size making it resectable (141). In general, multimodal therapy for recurrent HCC (TACE, local ablation and re-resection) might yield a 5-year survival rate of up to 20% (115, 138).

1.2 Liver regeneration and tumor recurrence

1.2.1 Post-operative Liver regeneration

Liver resection, and partial liver transplantation, as well as RFA, imply a post-procedural liver regeneration process. Liver regeneration is a reparative process following damage to the liver tissue or as a result of surgical resection. The growth of the remnant liver or graft leads to restoration of function by inducing hyperplasia of the liver remnant, but the removed lobes themselves do not regrow.

The process of restoration of liver volume in humans and rodents is initiated by replication of various types of intrahepatic cells (142-145). Hepatocytes are stable, highly differentiated cells of epithelial nature that rarely divide. Replication of hepatocytes generally starts within 1 day after major resection. The hepatocytes go from the quiescent G0 phase to the G1 phase and then undergo sufficient rounds of mitosis through the stimulation of growth factors and other

mitogens to restore the original mass of the liver. Nonparenchymal cells, such as endothelial cells, hepatic stellate cells (HSC), Kupffer cells, and biliary duct cells replicate in a delayed fashion, but demonstrate a similar synchronous pattern of DNA synthesis and mitosis as seen in hepatocytes. The interaction between mature hepatocytes and non-parenchymal liver cells and the most important growth factors are illustrated in Figure 2. From gene array and proteomic studies, it has been demonstrated that numerous genes are involved in liver regeneration. This activation pattern leads to alterations that fall into three main categories: release of cytokines, production of growth factors, and alterations in metabolic functions (146-150).

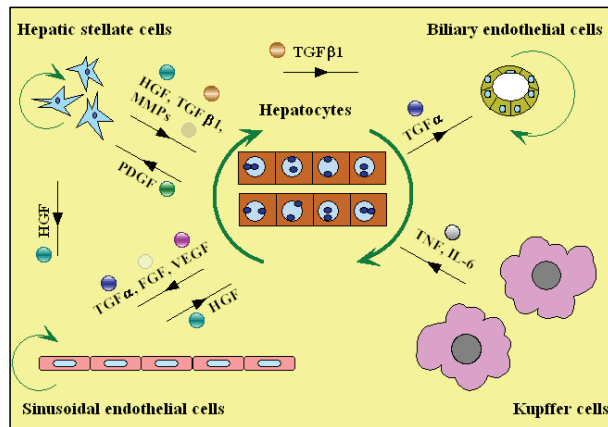


Figure 2. Molecule-mediated liver regeneration through interactions between mature hepatocytes and non-parenchymal liver cells (Kupffer cells, sinusoidal endothelial cells, biliary endothelial cells and stellate cells) (151).

EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IL-6: interleukin 6; MMPs, matrix metalloproteinases; PDGF, platelet-derived growth factor; TNF: tumor necrosis factor; TGF-α, transforming growth factor α; TGF-β1, transforming growth factor β1; VEGF, vascular endothelial growth factor

The progression of liver regeneration is highly coordinated by signal communication between hepatocytes and non-parenchymal cells, and is also influenced by endocrine glands, sympathetic innervations, and blood circulation. The progression of liver regeneration is

segmented into **three stages**: priming, proliferation and termination (Table 6). The first “priming stage” occurs within the first few hours after resection. The trigger of the regeneration cascade is thought to be the result of nitric oxide (NO) and prostaglandin (PGs) release induced by increased shear stress due to increased blood flow-to-liver mass ratio (152, 153). This initiation trigger is followed by an increase in liver cytokines, tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6), and the main cytokine-activated pathways are tightly controlled (149, 154, 155). Hepatocytes primed through these pathways become responsive to growth factors and enter into the second stage, the proliferative stage. The proliferative stage is dominated by growth factors and their receptors which have proliferative and cytoprotective functions. The main factors involved are HGF, epidermal growth factor (156) receptor ligands such as EGF and transforming growth factor alpha (TGF- α), amphiregulin, growth hormone (GH), and insulin-like growth factor (IGF) (144). The increased metabolic demand on the remaining liver remnant after damage or resection may be the sensor that dictates the extent of replication and also signals the termination onset. Proliferation inhibiting factors such as the transforming growth factor beta (TGF- β) superfamily is involved in the termination stage of liver regeneration(144, 157-159).

The cell proliferation is accompanied by breakdown and remodeling of the extracellular matrix (ECM) (157). During the proliferation phase, the hepatocytes form vascular clusters. The sinusoids become shorter and more dilated and completely disappear in some areas. Nonparenchymal cells become activated, proliferate later than hepatocytes and form clusters adjacent to the hepatocytes. In the later stages of regeneration, HSCs function as fibroblasts and secrete ECM components under the stimulation of TGF- β . Endothelial cells migrate into the hepatocyte clusters initiating a reorganization of the hepatocytes and formation of microcirculation. Angiogenesis involves ECM remodeling and the up-regulation of pro-angiogenic growth factors such as hypoxia-induced factor-1 α (HIF-1 α), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), metalloproteinases (MMPs) and basic fibroblast growth factor (b-FGF) (160, 161). New vessels are formed through proliferation and migration of endothelial cells from neighboring vessels and the mobilization and recruitment of hepatic progenitor cells (HPC) from bone marrow (162-164). The mobilization of

both types of cells is probably induced by local VEGF production, which is upregulated in liver regeneration (164).

Table 6. Sum-up of molecule-mediated liver regeneration (142, 151, 165)

Phases	Molecular factors	Origin	Targets & Effects	Signaling molecules
Priming stage	Nitric oxide, prostaglandins, tumor necrosis factor, interleukin 6	Nonparenchymal liver cells (NPLCs)*	Trigger of the liver regeneration, sensitizing hepatocytes to growth factors for replication	NF-kB, JAK/STAT3 and MAPK signaling pathway
Proliferation stage	Hepatocyte growth factor (HGF)	Hepatic stellate cells (HSCs) and other NPLCs, hepatocytes; mesenchymal cells	Cooperative effects allow the hepatocytes to overcome cell-cycle	HGF/cMet signaling pathway
	Transforming growth factor α (TGF- α)	Hepatocytes, HSCs	Checkpoint-controls and move hepatocytes from G0, through G1, to the S phase of the cell cycle, leading to DNA synthesis and cell proliferation.	EGFR signaling pathway IGF-1R and insulin receptor
	Epidermal growth factor (156)	Salivary glands in intestine and pancreas		
	Insulin-like growth factor (IGF)	Hepatocytes, NPLCs		
	Platelet-derived growth factor (PDGF)	Hepatocytes	Mitosis in NPLCs, remodeling of extracellular matrix (ECM)	PDGFR in HSCs VEGF receptor in endothelial cells
	Vascular endothelial growth factor (VEGF)			
Termination stage	Transforming growth factor β (TGF- β)	HSCs, other NPLCs, mesenchymal cells	Inhibition of hepatocytes DNA synthesis, remodeling of ECM, restoration at the end of regeneration	TGF- β signaling pathway
Other factors	<p>Wnt/β-catenin plays a supportive role in liver regeneration.</p> <p>Metalloproteinase play a pivotal role in ECM degradation, generation and degradation of active growth factor and signaling molecules in ECM.</p>			

*Nonparenchymal liver cells: Kupffer cell, sinusoidal endothelial cells and hepatic stellate cells

1.2.2 Factors in liver regeneration influencing tumor growth and metastasis

Several of the cellular and molecular changes resulting from hepatectomy and subsequent liver regeneration might influence the kinetics of tumor growth and thereby contribute to recurrence (Figure 2 and 3). A number of clinical and experimental studies have suggested that specific molecules essential for the regulation of liver regeneration may also promote the growth of residual dormant micrometastases postoperatively, leading to tumor progression (166, 167).

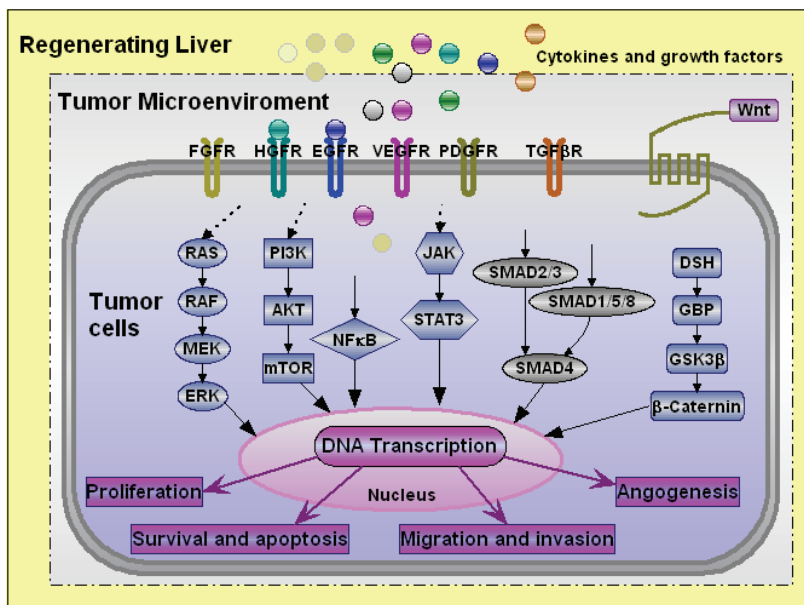


Figure 3. Signal pathways participating in the propagation of liver cancer during liver regeneration.

EGFR, epidermal growth factor/ transforming growth factor α receptor; FGFR, fibroblast growth factor receptor; HGFR, hepatocyte growth factor receptor; PDGFR, platelet-derived growth factor receptor; TGF β , transforming growth factor beta receptor; VEGFR, vascular endothelial growth factor receptor. NF κ B and JAK/STAT3 pathways, Phosphatidylinositol-3 kinase (PI3K)/AKT pathway, RAS/RAF/MEK/ERK pathway, TGF- β /SMAD pathway, WNT/ β -catenin pathway and activation of nuclear transcription are implicated in this process.

Micrometastases or de novo cancers may remain dormant for a long time when the proliferation and apoptosis rates of the tumor cells are mutually antagonistic (168, 169). In addition, angiogenic inhibitors such as circulating angiostatin, ECM proteins such as thrombospondin, and ECM protein fragments such as endostatin are considered important for maintaining a dormant state (170, 171). Tumor growth requires the balance of growth factors and cytokines in the microenvironment to favor angiogenesis (172).

Postoperative liver regeneration leads to release of cytokines and angiogenic factors such as IL-6, TNF- α , HGF, EGF, TGF- α , TGF- β , HIF-1 α and VEGF. This can alter the microenvironment of dormant tumor cells and cause tumor cell proliferation, migration and angiogenesis, and thereby contribute to increased tumor development (144, 149, 157, 173-182). Endocrine, autocrine and paracrine mechanisms activated during liver regeneration might also influence the growth of liver tumor cells (144, 183). Usually, pathways that inhibit HCC cell growth also impair liver regeneration. For instance, liver regeneration is regarded as an angiogenesis-dependent phenomenon (184, 185). Hepatocellular production of VEGF shows the maximal levels between 48 and 72 h after partial hepatectomy, and can increase dilatation and permeability of blood vessels (186). Inhibition of angiogenesis with angiostatin impairs both angiogenesis and liver regeneration (184).

Liver ECM breakdown and rebuilding is another major feature of tumor progression where tumor cells may become detached and enter into the blood and lymphatic circulation. The up-regulation of factors, which are common to liver ECM remodeling during liver regeneration, involving urokinase-type plasminogen activator (uPA), MMPs, HIF1 α and VEGF, are also activated at the tumor interface leading to tumor progression and invasion. In addition, metastatic epithelial tumors may undergo an epithelial to mesenchymal transition (EMT) through various stimuli in the tumor microenvironment leading to increased invasiveness (187). These stimuli include tyrosine-kinase receptors (TKR) ligands, such as b-FGF, EGF, HGF, TGF- β superfamily and MMPs (188).

1.2.3 Liver regeneration and hepatic progenitor cells

In the normal adult liver loss of liver parenchyma is repaired by replication of mature hepatocytes as outlined above (189, 190). However, pre-existing liver diseases such as chronic HBV and HCV infection, alcoholic fatty liver disease, hemochromatosis and other conditions may severely impair the ability of hepatocytes to replicate, promoting a second regenerative pathway through the activation of hepatic progenitor cells (HPCs) (151, 191). HPCs (also called oval cells in rodents) are quiescent, existing in low numbers around the periportal region (the canals of Hering) in normal livers. Following severe and prolonged liver trauma, these cells are capable of proliferation and differentiation into both hepatocytes and cholangiocytes in order to restore liver mass, and typically form the hepatoblast comprising small ductules and strings of cholangiocytes, termed the ductular reaction (191). HPC are present primarily in the portal tracts and extend into the parenchyma expressing both hepatocyte and bile ductular markers, as well as certain neuroendocrine markers. These HPC have been referred to as facultative stem cells or as a reserve stem cell compartment (150). Some studies have suggested that the presence of HPCs might also contribute to liver regeneration following transplantation (192, 193). HPCs may be of great importance to maintain and restore liver homeostasis under diverse situations like significant liver diseases or major liver surgery. .

Another source of cells with stem like properties is hematopoietic stem cells (HSCs). These cells may be recruited from the bone marrow by chemo-attractants, and then migrate and infiltrate the liver lobules via the canals of Hering. Activation of HSCs after hepatic surgery appears to be related to the extent of resection and the presence of concomitant liver disease (194). Liver resident stem cells may be another source of HPCs. Hepatocellular damage initiates an immune response in the liver, which leads to the secretion of a complex mixture of cytokines and growth factors. TNF- α and IL-6 released by Kupffer cells may stimulate HPC proliferation, while interferon (IFN) can prime HPC to respond to mitogenic stimuli. HPC growth factors released by HSCs include EGF, TGF- α , HGF and TGF- β . Thus, the cytokines released by Kupffer cells, hepatic stellate cells and HPCs themselves may act in concert to control HPC proliferation and remodeling of the liver parenchyma (189, 195, 196) .

Several studies have reported that liver resection and acute liver injury under certain conditions can lead to mobilization of progenitor like cells in the liver (162, 191, 197-199). In a recent study, Langenberg et al (198, 199) showed that major liver resection resulted in mobilization of HSCs into liver and further differentiation into HPCs. This was preceded by elevated levels of granulocyte colony-stimulating factor (G-CSF), indicating G-CSF may participate in the activation of HPC. Both the hematopoietic derived and the hepatic HPCs require essential inflammatory cytokines and regenerative growth factors to proliferate and transdifferentiate. These include TNF, IL-6, IFN- α , TGF- α , FGF, TGF- β and HGF (196, 200). The activation and differentiation of HPC is probably a delicate regulatory interplay between liver stress and regenerative response, but our knowledge of how and under which conditions progenitor cells are activated during human liver regeneration are still very limited.

1.2.4 Hepatic progenitor cell and HCC development

It has been suggested that HPCs may contribute to hepatic carcinogenesis through their particular activation in liver regeneration (201-203). This hypothesis has been supported by experiments in both rodents and humans. In human chronic liver diseases, especially HBV or HCV infection as well as liver cirrhosis, proliferation of HPCs are directly related to disease severity. This could suggest that activation of HPCs is associated with increased risk of HCC development in chronic liver disease (204). The fact that many human HCC tumors contain a mixture of differentiated hepatoma cells and an intermediate phenotype similar to HPCs, have introduced the concept that some hepatocellular cancers might arise from HPCs (201, 205, 206). Furthermore, these tumors exhibit gene expression profiles identical to hepatoblasts. In some studies it has been proposed that 28-50% of human HCC tumors may be derived from HPCs, and retrospective analyses have revealed that these tumors are associated with a significantly poorer prognosis and a higher recurrence rate after surgical resection and liver transplantation (201, 205, 206). Furthermore, recruitment of HPC has been linked to development of both HCC as well as intrahepatic cholangiocarcinoma in preclinical models. All these findings underline the significance of these cells in the initiation and progression of primary liver malignancies (151,

202, 203, 207, 208). It has also been suggested that liver cancer stem cells are probably derived from HPCs (208-210).

HPCs might also influence HCC progression through the contribution of growth factors. One study suggested that activation of HPCs in non-tumorous liver tissue was associated with recurrence of combined hepatocellular-cholangiocarcinoma after surgery (211).

2. AIMS OF THE STUDY

Based on the results in the literature, it might be hypothesized that HCC recurrence after surgery is influenced by cellular and molecular changes involved in postoperative liver regeneration. This could possibly stimulate the growth of occult tumors or dormant micro-metastasis not diagnosed by preoperative imaging.

In order to investigate the effect and nature of postoperative liver regeneration on HCC growth, the following aims were defined:

- 1) To test the hypothesis that a microscopic HCC tumor in the setting of partial hepatectomy would show enhanced growth and signs of increased invasiveness corresponding to the size of the liver resection.
- 2) To explore the status of HPCs following major hepatectomy and tumor implantation in a rodent liver resection model, and to investigate how HPCs might affect the biological behavior of hepatoma cells and experimental HCC tumors *in vitro* and *in vivo*.
- 3) To investigate the effect of RAF targeted therapy on HCC growth and liver regeneration in an experimental model of liver resection and microscopic HCC tumors.

3. SUMMARY OF ARTICLES

Our knowledge of factors disposing for recurrence of HCC is incomplete and there is a lack of truly effective adjuvant treatments. Risk factors related to tumor biology, liver disorders and liver surgery, especially liver regeneration after surgery does most likely influence the kinetics of tumor growth and might contribute to recurrence.

The aim of this work was firstly to explore the relationship between liver regeneration after surgery and the growth of experimental HCC tumors in the liver. This was outlined in Paper I based on the observations from an experimental model of liver resection and microscopic HCC implantation. In paper II, the effect of HPCs and postoperative liver regeneration was studied in more detail. In paper III, a therapeutic strategy to inhibit HCC growth by targeting RAF/MAPK signaling pathway was studied.

3.1 Article I

Liver resection of varying size influences the growth and malignant properties of experimental HCC tumors and the effect is correspondent with the size of the partial hepatectomy.

In this study, varying degree of partial hepatectomy was performed in groups of Buffalo rats with the concomitant implantation of a fixed number of hepatoma cells in the remnant liver. A control group underwent only resection. After 21 days, tumor size and number as well as the expression of AFP, Ki-67, cyclin D1, calpain-small subunit 1 (CAPNS1), CD34 (a microvessel density marker), Vascular Endothelial Growth Factor (VEGF) and its receptor 2 (VEGFR-2) were evaluated and compared among the groups. The results showed that the tumor volume and number increased significantly with the size of the partial hepatectomy ($P<0.05$). The largest resections were also associated with increased hepatoma cell infiltration in the lungs and significant upregulation of Cyclin D1, α FP, Ki67, CAPNS1, CD34, VEGF and VEGFR-2, suggesting a more malignant transformation of the tumors in the largest resections.

3.2 Article II

Hepatic progenitor cells stimulate tumor cell growth and promote a malignant transformation towards a more invasive phenotype with expression of stem-like properties.

In the paper I, It was noted that animals with tumor implantation that underwent major hepatectomy

displayed an enhanced liver regeneration as compared with animals that had major liver resection alone. Thus, it was hypothesized that the combination of major liver resection and tumor implantation might lead to activation of progenitor like cells. In paper II, activation of progenitor like cells was found in the regenerating liver after 70% hepatectomy and concomitant tumor implantation in both Buffalo and Fischer 344 rats by double immunofluorescence staining for cytokeratin 19 and α FP/CD133.

The effects of HPCs (WB-F344) on hepatoma cells (JM1) *in vitro* were investigated by a co-culture system with the conditioned medium technique (212) and proliferation rate, sensitivity to Adriamycin and expression of factors linked to invasiveness were assessed. The *in vivo* effect of HPCs on tumor growth was tested in Fischer 344 rats undergoing 70% hepatectomy and implantation of either naive JM1 cells, naive JM1 cells with syngeneic HPCs infusion or co-cultured JM1 cells.

Co-cultured JM1 cells showed more malignant properties by an increased IC₅₀ after Adriamycin exposure, and enhanced expression of α FP, MMP9, CD133, ABCG2, and pERK, pAKT, pSMAD2, β -catenin and anti-active β -catenin expression. Animals with JM1 tumors and concomitant syngeneic HPC (WB-F344) infusion or tumors induced by co-cultured JM1 cells displayed markedly increased tumor size and metastatic rate, suggesting that the presence of HPCs leads to a more aggressive phenotype. This could possibly partly be linked to TGF- β induced epithelial mesenchymal transition of tumor cells.

3.3 Article III

RAF targeted therapy inhibits tumor proliferation and metastasis without retarding liver regeneration as a result of differentiated responses of primary hepatocyte and hepatoma cells to RAF/MAKT inhibition.

Sorafenib (RAF targeted inhibitor) was applied to cell culture systems for primary hepatocytes and hepatoma cell lines. The cell viability was assessed with MTT assay, DNA synthesis with [³H]-thymidine incorporation assay and RAF/MAKT kinase signaling pathways were investigated by western blotting. Buffalo rats with and without hepatoma cell implantation after 70% hepatectomy received daily oral gavages of Sorafenib at a dose of 2.5/10 mg/kg from day 3 post surgery. At termination of the animal experiment at Day 21, tumor size, number as well as the markers Cyclin D1 and α FP in groups with and without Sorafenib treatment were compared.

Liver integrity was evaluated by liver function tests and hepatocyte proliferation was assessed by measurement of the Cyclin D1 and Ki-67 in the normal liver tissue.

The in-vitro studies showed that rat primary hepatocytes displayed higher cell viability, proliferation rate and stronger activation of RAF/MAKT kinase compared with hepatoma cell lines when the cells were exposed to Sorafenib. The tumor volume, number and rate of metastatic pulmonary cell infiltration after 70% partial hepatectomy were all decreased following treatment with Sorafenib ($P<0.05$) and no significant change in liver regeneration was found in the *in vivo* experiments ($P>0.05$).

4. DISCUSSION

4.1 Methodological considerations

The following methodological considerations will discuss the choice of methods and experimental model, and try to address the most important advantages and limitations.

4.1.1 Experimental model

Partial hepatectomy (70%) in rats have been widely used to study liver regeneration, acute liver failure, tumor dormancy of hepatic metastasis, hepatic function and response to stress and trauma in many previous studies(154, 183, 213-222). Resection models have also been used in studies of liver metastasis after direct hepatic inoculation or after intraportal or intrasplenic injection of tumor cells (217, 223). The classical 70% hepatectomy model in rats is one of the best described and extensively studied experimental models in liver surgical research. Because each lobe has its own pedicle containing a portal triad, hepatic resections of various extents are simple and highly reproducible.

To establish the experimental model of HCC micrometastasis after liver surgery and to further examine the possible relationship between various degrees of liver regeneration and growth of the “occult” HCC, we used varying sizes (0-80%) of hepatectomy in rats together with implantation of syngeneic HCC cells throughout the series of experiments. The vessel-oriented microsurgical technique was used to reduce the remnant liver size in situ according to the modified technique of Rodriguez (224). So far the hepatectomy ranging from 5 to 95% of total liver weight can be easily performed with high reproducibility because of a fairly constant parenchymal mass of each lobe and the high precision of this vessel-oriented microsurgical technique employed. Intrahepatic HCC tumor was established by direct implantation of moderate quantity of hepatoma cells (McA-RH7777 and JM1 re-suspended in phosphate buffer solution) into the inferior right lobe of the liver remnant using a 27-gauge needle. Intraportal injection was avoided due the possibility of thrombotic complications and less predictable and standardized tumor formation. Tumor formation in the animals at 2-3 weeks after hepatectomy

was 100%, and the mortality rate associated with the procedure was less than 5%. One disadvantage with tumors created by direct inoculation is that they are artificial and not spontaneously forming. The biological behavior of these tumors might consequently not fully correspond to naturally occurring malignant tumors.

4.1.2 Cell proliferation

Cell proliferation has been utilized to study cell viability, cell signaling and cell cycle regulation. Several methods have been employed to explore the proliferation of primary hepatocytes and hepatoma cells in paper I, II and III.

The MTT-assay is a simple and frequently used method for assessment of cell viability and cell proliferation. The soluble yellow tetrazolium dye (3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide) is reduced intracellularly to an insoluble purple product. The crystals are solubilized with DMSO and measured spectroscopically. The reduction of MTT occurs at two sites in the mitochondrial electron chain (coenzyme Q and cytochrome C). However, MTT reduction can also occur within mitochondria endosomes and lysosomes. Reduced MTT formazan is transported to the cell surface through exocytosis (225).

Thymidine incorporation was used to quantify DNA synthesis in paper III. The method is frequently used to measure the amount of cells in S-phase during cell proliferation. By relating DNA synthesis (H^3 –thymidine incorporation) to total protein content of the measured cell culture dish, it reduces the possibility that loss of the dead cells affected proliferation data. The high specificity of immunological methods, based on recognition between antigen epitopes and antibody, were used to study relative protein levels and protein phosphorylation for specific markers. Cyclin D1 is a marker of the transition from G1 to S phase in the cell cycle, whereas the Ki-67 protein is present during all active phases of the cell cycle (G_1 , S, G_2 , and mitosis). We used semi-quantitative scores of Ki67 positivity on the histological sections and evaluated Cyclin D1 expression by Western Blotting for both hepatic cell proliferation and tumor cell growth.

4.1.3 Identification of progenitor like cells during liver regeneration

HPCs are bi-potential stem cells residing in human and animal livers that are able to differentiate towards hepatocyte and cholangiocyte lineages (226-228). This compartment has been called the progenitor (in humans) or the oval cell compartment (in rodents) and it resides in the canals of Hering. There has been a heated controversy for more than 20 years regarding the role of, or even the existence of HPCs in humans. Recent data have, however offered evidence for a progenitor or precursor role for HPCs.

Liver regeneration after partial hepatectomy does not normally involve hepatic or hematopoietic stem cells but depends on the proliferation of hepatocytes (189, 229).

Transplantation and repopulation experiments have demonstrated that hepatocytes, which are highly differentiated and long-lived cells, have a remarkable capacity for multiple rounds of replication (190). When the replicative capacity and function of hepatocytes is impaired, potential stem cells are activated. Such circumstances can be observed in cirrhosis or chronic inflammatory liver diseases, and some experimental rodent models. Activation of the progenitor cell compartment, also known as “ductular reaction”, can lead to cellular differentiation into hepatocytes and biliary cells (Figure 4)(191).

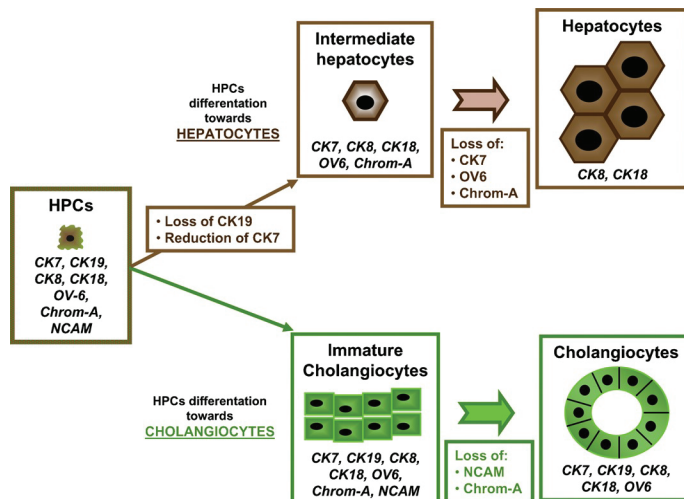


Figure 4. Differentiation of hepatic progenitor cells towards mature hepatocytes or cholangiocytes. This process is characterized by the appearance of trans- amplifying populations represented by intermediate hepatocytes and reactive ductules (191).

There is no single marker that offers complete specificity for the identification of hepatic progenitor cells (190). The stem compartment of the liver is assumed to consist of cells with various phenotypes and multiple molecular markers. In multiple separate studies, most of the markers have been identified by immunostaining in experimental models of HPC activation. These HPCs have been shown to share molecular properties with adult hepatocytes (albumin, cytokeratin 8 and 18), bile duct cells (cytokeratin 7 and 19, OV-6 and OV-1), fetal hepatoblasts (α FP), and hematopoietic stem cells (Thy -1, Sca-1, c-kit and CD34). Consequently, a combination of multiple markers is needed in order to differentiate HPCs from other hepatic cells (Table 7) (189, 230).

Table 7. Hallmarks of hepatocyte and hepatic progenitor cell markers (189, 230)

<i>Adult hepatocyte marker</i>	Albumin, Cytokeratin 8 (CK8), CK18, α 1-Antitrypsin, Hepatocyte nuclear factor (HNF4), HBD.1, c-MET
<i>Fetal hepatocyte marker</i>	α -Fetoprotein (α FP), Delta-like protein (dlk), Aldolase A and C, c-MET, Cadherin 22, CD24, CD44
<i>Adult biliary marker</i>	CK19, CD7, CK14, OV-1, OV-6, Glutathione-S-transpeptidase (GST-P), γ -Glutamyl transpeptidase (γ GT), Muscle pyruvate kinase (MPK), A6, OC.2 and OC.3, Connexin 43, CX3C11,MUC1, Deleted in malignant brain tumor 1 (DMBT1)
<i>Adult hematopoietic marker</i>	Thy -1, Sca-1, c-kit , CXCR4, CD24, CD34, CD133
<i>Marker of Hepatic progenitor cell</i>	All the above

4.2 General discussion

Both liver resection and partial liver transplantation imply a postoperative liver regeneration process. Efficient liver regeneration is a prerequisite for successful surgical therapy. The size of

the intended resection or size of a partial liver graft and the regenerative capability of the liver are determining factors for choice of surgical strategy as well as postoperative prognosis. On the other hand, accumulating clinical and experimental evidence suggests that factors involved in liver regeneration may stimulate tumor growth postoperatively.

4.2.1 Major hepatectomy results in intense liver regeneration and stimulation of HCC growth

Our findings (Paper I) indicate that the growth and invasiveness of an experimental tumor is significantly influenced by the size of the hepatectomy and thereby the magnitude of regeneration. Tumors from extensive resections were larger, more numerous and displayed higher levels of the proliferation markers. The tumors in the these large resection groups also appeared to have a greater malignant potential, indicated by higher levels of biochemical markers of invasiveness, migration and angiogenesis as well as a more infiltrative diffuse growth pattern in the histological sections. The increased malignant potential induced by larger hepatectomy is further underlined by the observed changes in lung morphology within the groups. The lung sections of animals with tumor implanted showed signs of local ischemia at gross examination, and marked inflammatory changes by microscopy that increased by resection size. Furthermore, there were an increasing number of tumor cells in the lung of the high resection groups suggesting early distant hematogenous metastasis.

It is evident that liver regeneration after hepatectomy provides a microenvironment with many growth factors and cytokines that are favorable for tumor cell proliferation and propagation of the tumor through cell invasion, adhesion and angiogenesis (142, 231-233). Several growth factors and cytokines are implicated in liver regeneration (223, 234-237). Studies using isolated hepatocytes have identified of several hepatocyte growth factors such as HGF, EGF, TGFs, insulin and glucagon and the cytokines including TNF- α and IL-6 (142, 231-233). Growth factors and cytokines can accelerate liver regeneration through activation of pathways such as RAS-RAF-MEK and PI3k-AKT cascades. Adult hepatocytes and the other non-parenchymal cells, including endothelial cells, Kupffer cells, lymphocytes and stellate cells are responsible for the upregulation of these products when liver regeneration is required after surgery (142). This

normal up-regulation in the microenvironment can stimulate occult tumors since the same pathways are important for tumor cell proliferation, adhesion, survival (anti-apoptosis), metastasis and angiogenesis, particularly in the early stages of liver regeneration (238, 239).

We found that there was an enhanced expression of Cyclin D1 after a 70% hepatectomy with concomitant tumor implantation (Paper I). This was accompanied by elevated α FP expression in the regenerating livers. As early as 1975, Bloomer and Boss (240, 241) demonstrated a correlation between α FP and liver regeneration in animal and human experiments. The role of α FP expression in regeneration process was poorly understood until the concept of HPC was evolved (200, 242-244). It has been found that HPCs share genetic characteristics with fetal hepatoblasts (AFP), biliary epithelial cells (CK19) and hematopoietic stem cell (CD133) and the combination of these markers could possibly be used to differentiate HPC from other cells within liver.

Our results (Paper II) suggest the HPC can be activated after a 70% hepatectomy in the presence of tumor, but not after major hepatectomy alone. The classic models of HPCs activation such as 2-N-acetylaminofluorene (AAF) in combination with partial hepatectomy (AAF/hepatectomy) have demonstrated that the mitogenic stimulus of hepatectomy may stimulate HPC when hepatocyte-mediated regeneration is inhibited (245). One might speculate whether the presence of a rapidly growing tumor, in combination with a major hepatectomy leads to an analogue mechanism of impaired proliferative capacity of the liver remnant ultimately leading to HPC activation. Future studies are needed to confirm our observation, better describe the mechanisms and the time frame of these processes.

Once activated, HPCs may promote tumor cells growth and propagation, indicated by the increments in growth rate and metastasis observed in the *in vivo* experiments as well as the more stem-like expression profile seen in the *in vitro* studies. This highlights mechanisms that can provide a better understanding of tumor biology after liver surgery and form the basis for new therapeutic strategies.

4.2.2 The use of targeted therapy to inhibit HCC growth after surgery

Improvements in the understanding of the molecular basis of cancer have led to the development of targeted agents tailored to inhibit specific protein kinases involved in intracellular signal transduction pathways, which drive tumor progression and recurrence. Development of HCC is suggested to be a multistep process associated with an array of molecular changes (236, 238, 246, 247). The RAF family members, (A-Raf, B-Raf, and C-Raf or Raf-1), are highly conserved serine/threonine kinases of the MAPK pathway. Activation of the MAPK pathway induces cell proliferation, differentiation, migration and inhibition of apoptosis. RAF/MAPK signaling pathway, consisting of a cascade of major cellular kinases (RAF> MEK> ERK), is often aberrantly activated in HCC and has been shown to play a critical role in the development of HCC (248). RAF kinase is overexpressed in a high percentage of HCC tumors, and the RAF/MEK/ERK pathway can be activated by various important etiologic factors such as HBV and HCV infection in addition to mitogenic growth factors (249). The RAF proteins activate the MAPK pathway where inappropriate and/or persistent activation leads to abnormal differentiation, proliferation and apoptosis, and cancer development (250).

The pan-RAF inhibitor, Sorafenib, targets the RAF/MAPK signaling pathway at the level of RAF kinase, blocking MEK/ERK activation and thus inhibiting CyclinD1 expression. In the present study, the direct effect of RAF inhibitor Sorafenib on cell viability and proliferation was verified in rat primary hepatocytes, hepatoma cell lines and rodent HCC tumor model. The inhibitory effect of Sorafenib on tumor cell proliferation was in line with the literature (251, 252). Significant inhibition of cell viability and proliferation rate was observed in McA-RH7777, BEL7402 and HepG2 cells at a concentration between 5 and 10 μ M. Sorafenib treatment in the in vivo model also reduced tumor growth and number, angiogenesis response and microscopic tumor cell infiltration of the lungs.. Interestingly, there was no apparent inhibitory effect of Sorafenib treatment on hepatocyte proliferation or liver regeneration within the concentrations tested in this study. The drug levels are in line with current therapeutic recommendations (251, 252). One interesting finding of this study is that Sorafenib in the concentration below 10 μ M did not block MEK/ERK or inhibit CyclinD1 in rat hepatocytes stimulated with EGF. The hepatocytes even showed an enhanced proliferation profile compared with hepatoma cells and

the cell viability was considerably higher than without Sorafenib incubation. The observed levels of the liver regeneration parameters in the animal experiments also supported this finding.

Thus, the study indicates that RAF inhibitor Sorafenib can inhibit postoperative tumor growth and metastasis without retarding liver regeneration in an experimental setting. These results could promote the use of RAF/MAPK targeted therapy as an adjunct to surgery for HCC. The timing of such a regimen in relation to the postoperative course is most likely important to avoid adverse events on postoperative recovery. In the study, the medication was started at day 3. In rodents, the regenerative process is usually very fast and well established by this time. Our study does not indicate at what time it might be feasible to give Sorafenib in adjunct to liver resection in a similar human trial, but based on known physiological differences between humans and rodents as well as safety concerns it should be much later than in this particular experiment.

The enhanced proliferation rate observed in hepatocytes is in contrast to some experiments using selective MEK inhibitor (U0126), which reported inhibition of proliferation (253). RAF kinases generally participate in the RAS-RAF-MEK-ERK signal transduction cascade. The diverging effects are probably because MEK is the downstream kinase of RAF, and RAF has many interactions with other kinases, for example PI3K/AKT signaling pathway as was shown in paper III.

Different responses in the RAF/MAPK cascade between hepatoma cells and untransformed hepatocytes are probably due to gene mutations. The V600E mutation, is the most frequently (90% of cases) mutation found in B-Raf (oncologic RAF gene), and is associated with human cancers, especially in 30-60% melanoma and 30-50% thyroid carcinoma and 5-20% of colorectal cancers and ovarian cancer (254, 255). RAF inhibitors were found to prime wild-type RAF to activate the MAPK signaling pathway and enhance growth, while RAF inhibitors effectively block the MAPK pathway and decrease tumor growth in mutant B-RAF (V600E) tumors (256-258). Thus mutational status of B-RAF is thought to affect the sensitivity of tumor cell lines to Sorafenib (259). HCC is still regarded as regulated by C-RAF and few specific genetic or structural aberrations have been identified in HCC where C-RAF is activated in the absence of upstream

RAS activation (260). Therefore the observed effects of Sorafenib on hepatoma cells and primary hepatocytes were assumed to be attributed to diverse biological functions of RAF isoforms (256). However, we did not detect V600E mutation in the three hepatoma cell lines, suggesting that mutation is not frequently detected in HCC. Although our restricted sample size prevents us to make solid conclusions, this indicates B-RAF V600E mutation probably does not participate in tumor inhibition as in melanoma (257). Thus, the observed effects of Sorafenib on hepatoma cells and primary hepatocytes were not attributable to V600E mutation.

5. CONCLUDING REMARKS

Based on the results of the published papers, we have demonstrated:

- Microscopic HCC tumors in the setting of partial hepatectomy show enhanced growth and signs of increased invasiveness corresponding to the size of the liver resection.
- Activation of hepatic progenitor cells in the regenerating liver was observed after major hepatectomy with concomitant tumor implantation. The progenitor cells stimulate the hepatoma cells and contribute to a malignant transformation characterized by increased invasive capability in vivo and expression of stem-like properties in vitro.
- RAF targeted therapy can inhibit tumor proliferation and metastasis after surgery without retarding liver regeneration in an experimental setting. This was related to a differential response of primary hepatocyte and hepatoma cells to RAF/MAKT inhibition.

The results provide a better understanding of some of the aspects related to recurrence of HCC after liver surgery and could be of significance for liver resection and partial liver transplantation strategies for HCC.

Important principles are outlined in figure 5.

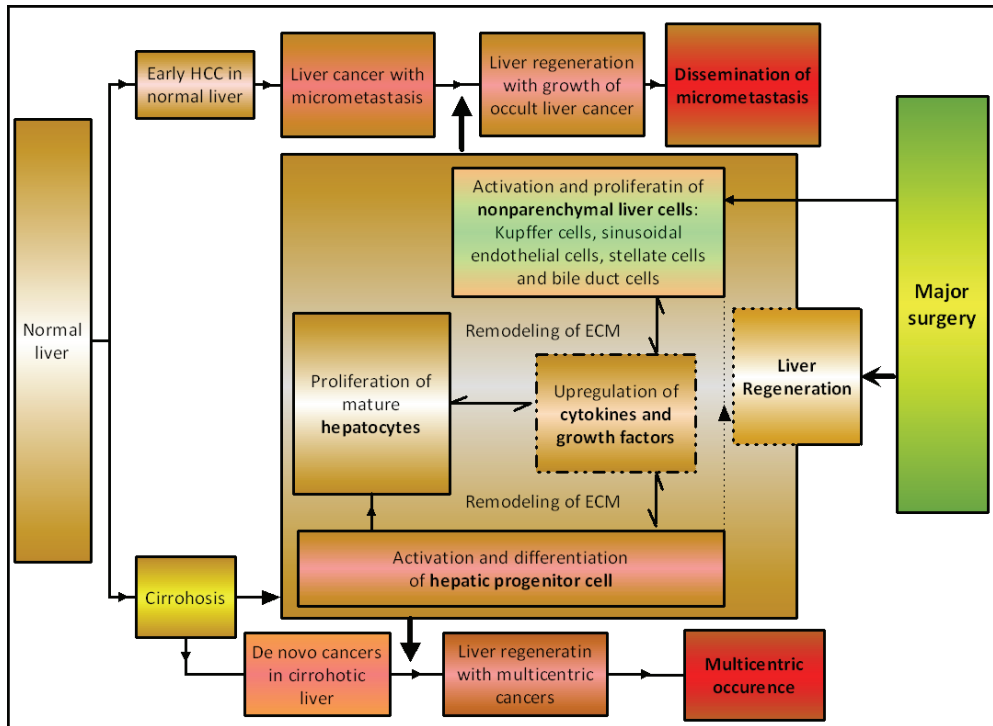


Figure 5. Liver regeneration and progression of remnant liver cancer or *de novo* cancer after liver surgery

HCC, hepatocellular carcinoma; ECM, extracellular matrix

6. FUTURE PERSPECTIVES

The work presented in the thesis provides a better understanding of basic mechanisms that could promote liver tumor recurrence after liver surgery. Nevertheless, a large number of questions implicated in this work are still uncovered and need further clarification.

Most surgical curative treatments for liver tumors induce postoperative liver regeneration. It would be of great interest to compare partial hepatectomy, reduced-size liver transplantation, portal vein ligation (PVL) and portal vein embolization (PVE) in both animal models as well as prospective clinical trials with respect to regeneration signal and relapse of tumor. Clinical studies within this field will probably need large sample sizes and a careful design. Some experimental and clinical research studies on PVL, PVE and LDLT have suggested that the regeneration associated with the treatment seems to influence tumor growth. Furthermore, it is justified to look into whether the biological principles outlined in this work are valid and relevant also for secondary liver tumors like colorectal liver metastases.

The role of progenitor cells in the development of HCC is an evolving theme that needs further studies. To what extent progenitor cells are activated after major liver surgery needs better characterization and how this might influence the prognosis of surgical therapy for HCC needs further scientific examination. Over 80% of HCC is complicated with liver cirrhosis and chronic hepatitis, where hepatic regeneration involves the recruitment of HPCs. More investigations on the mechanism underlying the activation of progenitor cells might lead to new therapeutic strategies that could improve the prognosis of surgical therapy.

More efforts should be focused at comparison of gene and protein expressions in normal hepatocytes and malignant cells by microarray and proteomic technology, as well as identification of signaling pathways that are unique to the hepatoma cells, in order to identify novel targets for treatment and to better understand the cell population that escapes the current treatment and causes recurrences.

Lastly but importantly, studies of tumor- immunology, which has not been a part of in this thesis, is critical for a better understanding of immune evasion of the tumor observed in liver cancer. The mechanisms that enable liver cancer to escape attack by the immune system still

remain fully unclear. Further knowledge into how immunogenic cell death of cancer cells might be evoked can aid in the development of novel therapeutic principles. Anti-tumor directed therapy is an evolving and promising future field that holds great promise and that possibly could lower recurrence rates and improve survival after surgery for liver tumors.

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APPENDIX: Paper I - III

