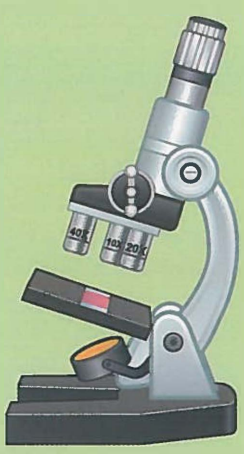
The microenvironment of the inflammatory type hepatocellular adenoma

Han, Jing



The Microenvironment of the Inflammatory Type Hepatocellular Adenoma

Jing Han

STELLINGEN

Behorend bij het proefschrift:

The Microenvironment of the Inflammatory Type Hepatocellular Adenoma

1. The liver in which the inflammatory type hepatocellular adenoma develops is not normal (This thesis).

2. The remnant liver of resected inflammatory type hepatocellular adenoma harbours foci of potential neoplastic growth (This thesis).

3. Fatty changes in the inflammatory type hepatocellular adenoma are secondary to the fatty constitution of the liver in which the tumor develops (This thesis).

4. The overweight/obese status of the patient contributes to the inflammatory phenotype of the inflammatory type hepatocellular adenoma (This thesis).

5. The ductular reaction in the inflammatory type hepatocellular adenoma is a hepatic-progenitor cell mediated regenerative response (This thesis).

6. Hepatocytes in cytochrome-*c*-oxidase deficient patches in the inflammatory type hepatocellular adenoma are generated by cells with stem cell properties, residing in the areas of ductular reaction (This thesis).

7. Subclonal expansion in the inflammatory type hepatocellular adenoma is a constitutive process regardless the neoplastic growth (This thesis).

8. Every honest researcher I know admits he's just a professional amateur. He's doing whatever he's doing for the first time. That makes him an amateur. He has sense enough to know that he's going to have a lot of trouble, so that makes him a professional. (Charles Franklin Kettering)

9. Tell a man that there are 300 billion stars in the universe, and he'll believe you... Tell him that a bench has wet paint upon it and he'll have to touch it to be sure. (Murphy's Law)

Jing Han, 2013

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To my parents

献给我的父母

Paranimfen:	Marian Bulthuis
	Ranran Li

CONTENTS

Abbreviations/Acronyms

AdiR2	adiponectin receptor 2
AMPK	5' AMP-activated protein kinase
BMI	body mass index
CCL20	CC chemokine ligand 20
CCO	cytochrome *c* oxidase
CRP	C-reactive protein
DR	ductular reaction
EC	endothelial cells
EpCAM	epithelial cell adhesion molecule
FNH	focal nodular hyperplasia
GNAS	G-protein alpha stimulatory subunit
GS	glutamine synthetase
HAEC	human coronary artery endothelial cells
HCA	hepatocellular adenoma
HCC	hepatocellular carcinoma
hHSEC	hepatic sinusoidal endothelial cells
HNF1α	hepatocellular nuclear factor-1α
HPC	hepatic progenitor cells
HSC	hepatic stellate cells
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IHCA	inflammatory type hepatocellular adenoma
IL-6	interleukin-6
IL6ST	interleukin-6 signal transducer
JAK2	Janus kinase 2
LFABP	liver fatty acid binding protein
LMD	laser microdissection
LR	leptin receptor
LRb	leptin receptor long isoform
mtDNA	mitochondrial DNA
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
PPAR-α	peroxisome proliferator-activated receptor-α
PPT	pseudo portal tracts
SAA	serum amyloid A
SDH	succinate dehydrogenase
SEC	sinusoidal endothelial cells
SOCS3	suppressor of cytokine signaling 3
Sox-9	sex determining region Y-box 9
STAT3	signal transducer and activator of transcription 3
TGF-β	transforming growth factor-β
TNFα	tumor necrosis factor-α
VAT	visceral adipose tissue
VEC	vascular endothelial cells

CHAPTER 1

Introduction and aim of the thesis

1.1 Benign hepatocellular tumors

Benign hepatocellular tumors are characterized by a proliferation of hepatocytes without invasion and metastasis. The two major lesions in this category are focal nodular hyperplasia (FNH) and hepatocellular adenoma (HCA). The incidence of hepatocellular benign tumors is low relative to other liver diseases. The prevalence of FNH was estimated around 0.4 to 3 per 100 of the general adult population based on two series of autopsy studies (1, 2). In contrast, the incidence of HCA is much lower, about 3-4 per 100,000 of the population with long term use of oral contraceptives (3).

FNH is a hepatocytic lesion with a star-shaped central fibrous scar with thick-walled vessels and radiating fibrous septa containing ductular reaction and inflammatory cells (4). These septa shape the nodularity of FNH which is a hyperplastic and non-neoplastic lesion. Its polyclonal nature has been established by Paradis et al (5). In 1985, Wanless et al presented a hypothesis on the origin of FNH that the abnormal proliferation of hepatocytes could be caused by vascular malformation leading to an increased blood flow focally (6). This theory has been supported by a study on the role of angiogenesis showing an increase of Angiopoietin-1/Angiopoietein-2 (Ang-1/Ang-2) ratio in FNH compared with other liver diseases including hepatocellular carcinoma (HCC) (7). The prominent role of Ang-1 in FNH has been corroborated by the results of a previous study in our center (8). Ang-1 has a significant role in hepatic vascular morphogenesis as has been shown in animal studies. Excessive and prolonged overexpression of Ang-1 resulted in abnormal vessel formation but also generated nodular parenchymal changes resembling FNH (9).

HCA is a benign neoplastic, monoclonal proliferation of well-differentiated hepatocytes arising in an otherwise non-diseased liver (10, 11). HCA consists mainly of hepatocytes without portal tracts, but randomly distributed solitary arteries and dilated sinusoids. Despite the lack of recent epidemiological data, the incidence of HCA is reported to be lower in Asian countries than in European countries (12, 13). Both FNH and HCA occur frequently in women during their reproductive age and there is an association between oral contraceptives and HCA

which is not conclusive for FNH (14-16). HCA has been regarded as a well-defined homogeneous entity until recent molecular investigations dissected HCA into subtypes, refining the further understanding of tumorigenesis mechanisms in HCA (17, 18).

1.2 Subtypes of hepatocellular adenoma (HCA)

A series of morphologic and genetic studies by the groups of Bioulac-Sage and Zucmann-Rossi resulted in a new genotype and phenotype classification of HCA (15, 17-19). According to this most recent classification, based on the morpho-molecular patterns, HCA is divided into 4 subtypes (Table 1): hepatocellular nuclear factor-1α (HNF1α) gene mutated type HCA, β-catenin gene mutated type HCA, inflammatory type HCA characterized by mutations of the interleukin-6 signal transducer (IL6ST) gene or signal transducer and activator of transcription 3 (STAT3) gene, and a group which lacks any of the mentioned mutations, the "unclassified" subtype.

HNF1α mutated (inactivated) adenomas account for 30-40% of all adenomas. Most of the mutations are somatic. Yet, patients with germline HNF1α mutations are even younger than those with somatic mutations, which are associated with maturity-onset diabetes type 3 (MODY3) and also are prone to have adenomatosis (20). Inactivation of HNF1α can lead to decreased or absence of liver fatty acid binding protein (LFABP) expression in the tumor cells, which is expressed in normal livers.

β-catenin mutated (activated) adenomas are found in 10-15% of HCA cases, which occur more frequently in males (21) and have more risk of HCC transformation than the other 3 subtypes. Glutamine synthetase (GS), one of the target genes of β-catenin activation, is usually strongly and diffusely overexpressed in β-catenin activated adenomas. Therefore an immuno-histologically diffuse pattern of GS expression on an HCA tissue sample can be used as an indicator of β-catenin mutation (15, 18). Of note, β-catenin mutation can also occur in the inflammatory type HCA (15, 22) and diffuse expression of GS, indicative of β-

catenin activation, has also been observed in one case of HNF1α mutated HCA with atypical morphological features (Gouw, unpublished observation).

Table 1 Classification of hepatocellular adenoma

Subtype	Gene mutation	Immunohistology	Frequency
Hepatocellular nuclear factor-1α (HNF1α) mutated type HCA	HNF1A	liver fatty acid binding protein (LFABP) (-)	30-40%
β-catenin mutated type HCA	β-catenin	β-catenin (+); glutamine synthetase (GS)	10-15%
Inflammatory type HCA	IL6ST* 60%	C-reactive protein (CRP) (+) serum amyloid A (SAA) (+)	40-50%
	STAT3* 8-12%		
	GNAS* 5%		
	Unknown 23-27%		
Unclassified type HCA	-	-	10%

* IL6ST, interleukine 6 signal transducer; STAT3, signal transducer and activator of transcription 3; GNAS, G-protein alpha stimulatory subunit.

1.3 The characteristics of the inflammatory type HCA

Among the 3 known subtypes, the inflammatory type HCA (IHCA) is a common type which accounts for around 40-50% of HCA cases in different centers (18, 23, 24).

Morphologically, IHCA is characterized by proliferation of near-normal hepatocytes similar to other types of HCA and also share the vascular changes such as solitary arterioles and dilated sinusoids. One morphologic characteristic that distinguishes IHCA from the other types is the presence of portal-tract like structures, also named pseudo portal tracts (PPT), in which several arterioles are present along with biliary ductules and inflammatory cells. Before the genetic background of this type of lesion was revealed, this HCA subtype used to be classified as telangiectatic FNH (25). The confusion with FNH is influenced by the presence of the PPT and biliary ductules. These structures are thought to be the characteristics of FNH since HCA has been regarded to consist of hepatocytes only. The distinctive immunophenotype of IHCA is the overexpression of the acute phase proteins C-reactive protein (CRP) and serum amyloid A (SAA) in the tumor cells regardless of the gene mutation types (15).

In IHCA, there is a somatic mutation of the IL6ST gene which encodes gp130 in 60% of IHCA, a somatic mutation of the STAT3 gene in 8-12% and 5% of IHCA was found to have G-protein alpha stimulatory subunit (GNAS) gene mutation (26-28). All of these mutations can directly or indirectly activate the STAT3 signaling pathway, leading to a "systemic inflammatory syndrome" in these patients, with increased serum CRP which decreased after tumor resection (29). Inflammatory type adenomas, like other HCA types, is a benign neoplasm but a concurrent β-catenin gene mutations can increase the risk of malignant transformation (30).

A pivotal clinical feature of patients with IHCA is the frequent association with an increased body mass index (BMI) (29, 31). Paradis et al reported that 60% of patients are overweight (mean BMI 28) in their studied population (29). The pathogenetic background of this association is not known.

Although the inflammatory phenotype in IHCA may result from IL6ST, STAT3 or GNAS gene mutations covering 73-77% of IHCA, in the remaining 23-27% no gene mutations are currently known that may explain the consistent over-expression of CRP and/or SAA and the inflammatory phenotype. Since overweight ($25 \leq BMI < 30$ kg/m^2)/obesity (BMI ≥ 30 kg/m^2) is known to induce an inflammatory state, the molecular background of a possible association between IHCA and overweight/obesity is one of the subjects of this thesis.

2. Microenvironment of IHCA

2.1 Obesity and tumorigenesis

Obesity or excess body weight has become a worldwide disease, leading to an increased risk of obesity-related tumors. Several gastrointestinal and hepato-biliary cancers are regarded as obesity-related tumors including colorectal adenoma and cancer, esophageal adenocarcinoma, pancreatic cancer, and HCC (32).

The mechanisms linking obesity with carcinogenesis have yet to be revealed, but it is certain that multifactorial interactions are involved. Insulin resistance and chronic inflammation are two obesity related processes that may be related with carcinogenesis. For example, in colorectal cancer, insulin resistance was shown to lead to free insulin-like growth factor 1 and 2 that bind to their corresponding receptors and stimulate cell proliferation by triggering the mitogenic and antiapoptotic cascades (33). Increased serum proinflammatory cytokines such as CRP and interleukin-6 (IL-6), representing a state of chronic inflammation in overweight individuals, are recognized to be positively associated with an increased risk of colon cancer and colorectal adenoma respectively (34, 35). The third possible mechanism involves adipokines derived from adipose tissues which will be addressed in **2.1.1**.

In liver diseases, obesity is associated with nonalcoholic fatty liver disease (NAFLD), and nonalcoholic steatohepatitis (NASH). The latter is clinically associated with the metabolic syndrome and is histologically characterized by steatosis and inflammation. NASH may eventually develop into cirrhosis. To some

extent, NASH is more prone to HCC transformation than NAFLD and the progression of NASH to HCC does not necessarily include a cirrhotic stage (36). Moreover, Park et al demonstrated that dietary and genetic obesity could induce HCC in mice via chronic inflammation involving, e.g., elevation of IL-6 and tumor necrosis factor (TNF) (37). In HCA, although only β-catenin (activated) mutated HCA has been identified to have an increased risk for HCC transformation, Farges et al demonstrated that there is a significantly increased frequency of malignant transformation of HCA into HCC in 59% of overweight male patients without β-catenin mutation (38). In IHCA patients, the increased serum CRP levels and their high BMI indicate an inflammatory state which may be associated with obesity, but the pathological mechanisms of this association are not clear yet.

2.1.1 Adipokines in liver disease

The term "adipokines" represents adipose tissue derived cytokines and includes several kinds of polypeptide factors generated by adipose tissues. An important role is played by ectopic fat in overweight individuals, representing dysfunctional adipose tissue, e.g., visceral adipose tissue, which produces adipokines that can target the liver directly via the portal vein. Overweight or obese individuals have more dysfunctional adipose tissues including visceral adipose tissue and subcutaneous adipose tissue. Both types could generate adipokines, such as leptin, adiponectin, resistin, TNFα, IL-6, monocyte chemoattractant protein-1, transforming growth factor-β (TGF-β), and plasminogen-activator inhibitor-1 (39), which are involved in among others regulating metabolic control, stimulating inflammation, and tissue repair.

Adiponectin, coded by the *adiponectin* gene, is the second product of adipose tissue, of which the concentration is inversely correlated with BMI (39-41). The structure of adiponectin is composed of multimers, generating three multimetric forms (42). All three forms circulate in the blood and bind two specific receptors, AdipoR1 and AdipoR2 (AdiR2). Skeletal muscle and liver are the main targets of adiponectin (43). AdipoR1 is abundantly expressed in skeletal muscles and AdiR2 is mostly expressed in the liver (44). Adiponectin can activate the 5'

AMP-activated protein kinase (AMPK) and the peroxisome proliferator-activated receptor-α (PPAR-α) signaling pathway to mediate fatty acid oxidation and glucose uptake in muscles and liver tissues (45). Adiponectin-AdiR2 signaling exerts "preventive" roles in liver, such as anti-steatosis by stimulation of hepatic fatty acid oxidation, anti-inflammation through inhibition of hepatic production of proinflammatory cytokines (TNFα), induction of anti-inflammatory cytokines (IL-10) in Kupffer cells and anti-fibrosis via attenuation of the effects of TGF-β in hepatic stellate cells (HSC) (46, 47).

Leptin, the main product of the adipose tissue, coded by the *obese* (*ob*) gene, is a 16kDa cytokine/hormone. It is well known as the key mediator regulating food intake and energy expenditure via the hypothalamo-pituitary-adrenal (HPA) axis. Serum leptin is positively related with increased BMI. Leptin receptor (LR) is expressed on vascular endothelial cells, sinusoidal endothelial cells (SEC) and some cells of the immune system besides the expression on the blood-brain barrier composed of central microvessels and neurons of the hypothalamus (39, 48, 49). LR, a member of the class I cytokine receptor family, has six isoforms (*a* to *f*), and is classified into a secreted (*e* isoform), short (*a*, *c*, *d*, and *f* isoform) and long isoform (*b* isoform). Of these, the long isoform (*b* isoform; LRb) is the only functional isoform with intracellular signaling. After binding with leptin, the intracellular domain of LRb will activate Janus kinase 2 (JAK2) phosphorylation, which leads to autophosphorylation of three other tyrosine residues (Y_{985}, Y_{1077} and Y_{1138}) at the tail of the LRb intracellular domain. Phospho-Y_{1138} can activate STAT3 which translocates into the nucleus and mediates the expression of downstream genes, including suppressor of cytokine signaling 3 (SOCS3). This will inhibit the signaling of LRb-JAK/STAT3 as a negative feedback. Phospho-Y_{1077} can recruit STAT5 and phospho-Y_{985} can promote the tyrosine phosphatase adapter protein SHP-2 (Figure 1) (50).

Leptin-LR signaling exerts an "aggressive" role in the liver. Characteristic hepatic steatosis can be observed in leptin-deficient (*ob*/*ob*) mice. Yet, fatty liver and hyperleptinemia are present in obese patients at the same time which is due to leptin resistance (39). Leptin resistance is the failure of high levels of serum leptin to suppress food intake and prevent or mitigate obesity in obese individuals (50,

51). Leptin has multiple effects on the liver and has been reported to be proinflammatory, fibrogenic, angiogenic and also stimulates hepatocytic proliferation and migration. The proinflammatory effects of leptin that have been documented include increased white blood cell counts which are positively related with serum leptin level in obese people (52), and leptin could stimulate TNFα production by circulating monocytes (53). Leptin has also been described as a profibrogenic factor by activating Kupffer cells to secrete TGF-β and targeting HSC (46). Upregulation of vascular endothelial growth factor induced by leptin on endothelial cells and HSC could promote angiogenesis in the liver (54, 55). Leptin supplementation stimulates liver regeneration in *ob*/*ob* mice after CCl_4 injury (56). Besides roles in angiogenesis and proliferation, leptin could promote migration and invasion of HCC cell lines (40), which may suggest that high levels of serum leptin could contribute to a favourable environment for tumorigenesis.

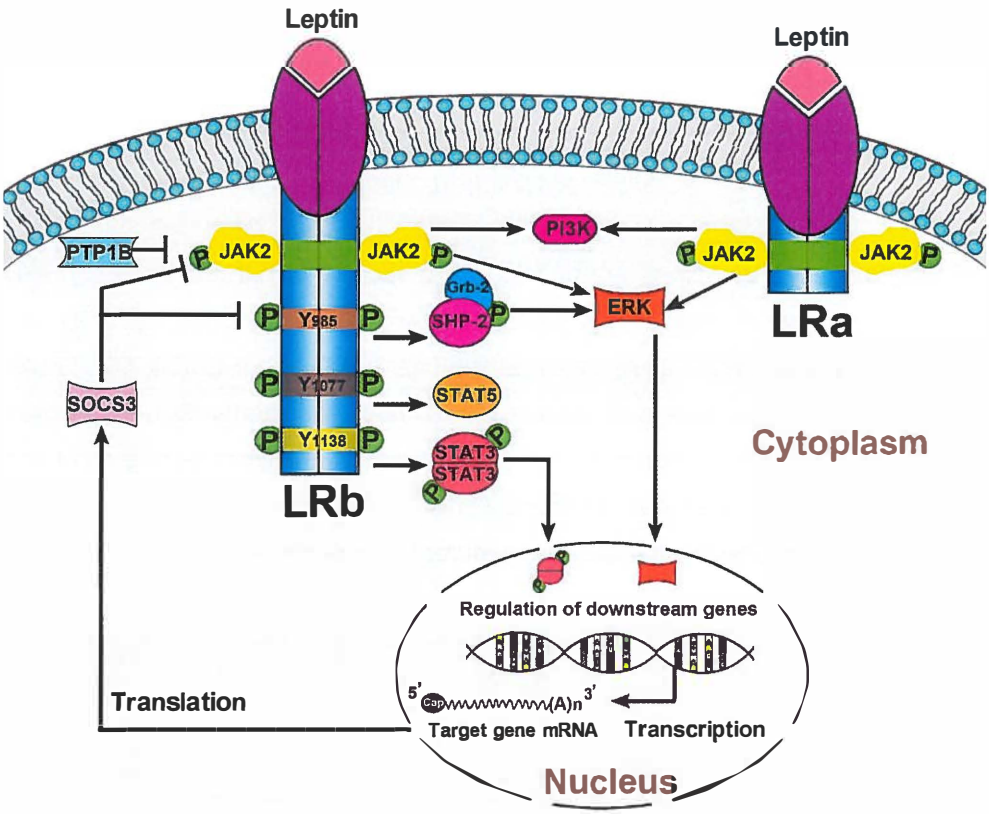

Figure 1 Pathway of leptin-leptin receptor induced signal transduction and feedback inhibition. LRb, long form of leptin receptor; LRa, short form (*a*, *c*, *d*, and *f*) of leptin receptor. After leptin binding to the leptin receptor, both isoforms can activate JAK2 phosphorylation, which only initiates autophosphorylation of three tyrosine residues (Y_{985}, Y_{1077} and Y_{1138}) on the intracellular domain of LRb. Tyrosine-protein phosphatase 1 B (PTP1B) and suppressor of cytokine signaling 3 (SOCS3) can inhibit LR/JAK2 signalling, the later representing a direct feedback loop downstream of leptin/LRb signaling.

2.3 Inflammation in IHCA

Inflammation in IHCA presents in two ways. IHCA, unlike the other types of HCA usually contains variable quantities of mixed inflammatory cells (Figure 2) consisting of predominantly of T lymphocytes of the CD4 and CD8 subtypes, few plasma cells, CD68+ histiocytes, and few neutrophils (26). Secondly, IHCA is

usually classified by the immunohistological overexpression of the acute phase proteins CRP and/or SAA according to the new classification (15). The molecular background the regulation of these two proteins in tumor tissue are the mutations including IL6ST gene mutation, STAT3 gene mutation, and GNAS gene mutation, all of which could activate the STAT3 signaling pathway without IL-6 binding. IL6ST mutation may also robustly upregulate CC chemokine ligand 20 (CCL20) gene expression in the tumor. CCL20 is a chemokine that directs chemoattraction of inflammatory cells such as lymphocytes and dendritic cells (26), which may represent one of the mechanisms leading to inflammatory infiltration in IHCA. However, as has been alluded before, the association of IHCA with obesity may also contribute to an increased inflammatory state in IHCA.

2.4 Ductular reaction in IHCA

Ductular reaction (DR) is defined as a reaction of ductular phenotype, possibly but not necessarily of ductular origin and is frequently present in many acute and chronic liver diseases (57). DR can have different patterns and lead to different consequences depending on the type of hepatic injury. As DR contains bipotential hepatic stem/progenitor cells, DR can generate mature hepatocytes and biliary epithelial cells. Hence, DR can take part in the replenishment of cellular loss by generating mature hepatocytes when the proliferative capability of mature hepatocytes is exhausted (58). Besides parenchymal regeneration, DR has been reported to induce fibrosis in many chronic diseases, and may also contribute to tumorigenesis. Biliary ductules are frequently present in IHCA but are rarely seen in the other HCA-subtypes. Whether these ductules represent DR as in other non-tumorous liver diseases is not known. Morphologically the ductules in IHCA show histologic resemblance with the non-tumorous DR because in IHCA the ductules are CK7 and CK19 positive and are located in the margin of the PPT, at the interface with the neoplastic hepatocytes (Figure 2). In this thesis the nature of DR in IHCA is characterized and the relation of DR with the neoplastic hepatocytes is addressed.

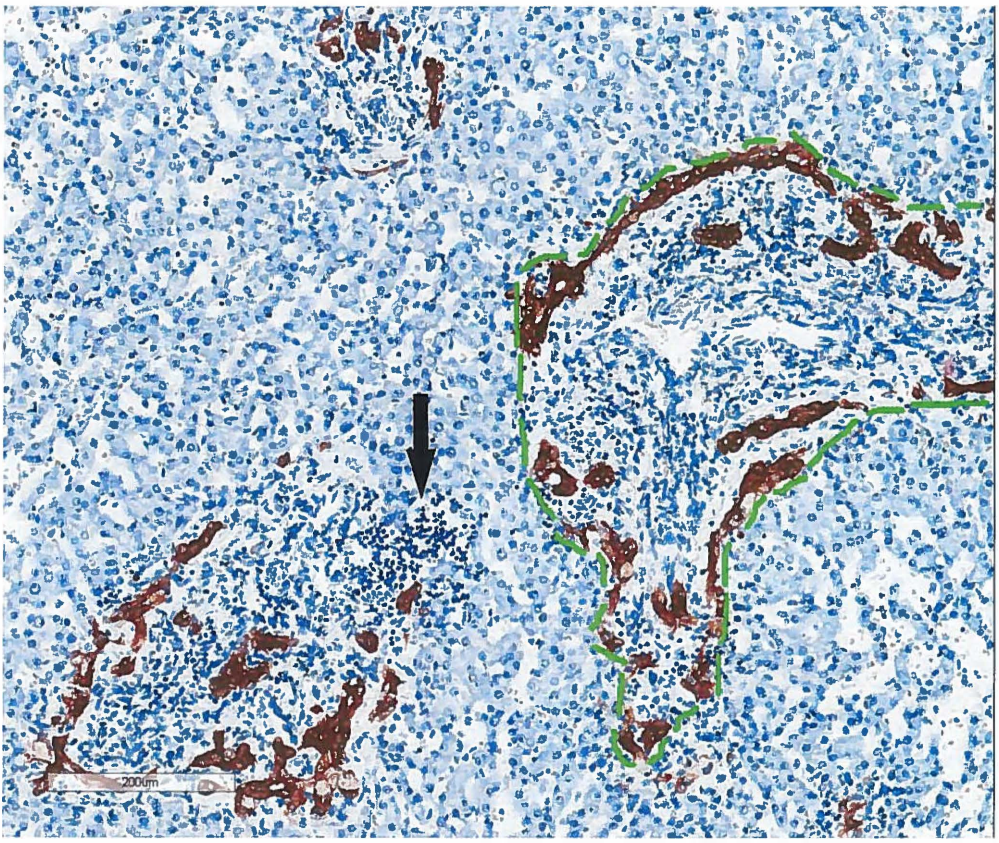

Figure 2 Ductular reaction in the pseudo portal tracts (PPT) of the inflammatory type hepatocellular adenoma (IHCA) highlighted by CK19 staining (brown) 50×. Ductular structures are predominantly located at the interface (green dashed line) of PPT stroma and the neoplastic hepatocytes. Arrow: a cluster of inflammatory cells.

3. Aim and scope of the thesis

Since obesity has been regarded as the aggressive factor for tumorigenesis, and IHCA patients show an association with high BMI, it is possible that over-weight/obesity may have a contributory role in the development of IHCA. Moreover, obesity may continuously provide a favorable environment for tumor-growth even after IHCA patients underwent tumor resection which may have consequences in their remnant liver. We hypothesized that a link exists between overweight/obesity

and the pathogenesis of IHCA. In the following chapters, we focused on the microenvironment in which HCA develops: the "normal" liver, the status of the adipokines receptors in the tumors and corresponding non-tumorous counter parts, the consequences of leptin stimulation on endothelial cells and the interplay between endothelial cells and leukocytes in *in vitro* assays, and the nature of the biliary ductules in IHCA.

It is generally assumed that HCA, unlike HCC, develops in an otherwise normal liver. However, IHCA is related with obesity which is a systemic disorder which may result in pathological changes in the non-lesional liver part of IHCA. Therefore, in **chapter 2** we investigated the features of the non-lesional liver parts of IHCA livers both histologically and immunohistologically. We studied changes related with obesity and the presence of features that characterize IHCA. Presence of both types of pathological features may have clinical consequences, such as the clinical follow-up management because these pathological changes would also be present in the remnant liver after resection of IHCA.

Chapter 3 addresses the influences of leptin and adiponectin in IHCA, representing the main types of adipokines linked with obesity. In this chapter, we examined the gene and protein expression of leptin, adiponectin and their corresponding receptors in IHCA, and tried to gain insight into the downstream effects of the leptin-LR signaling.

Overweight/obese individuals are known to have hyperleptinemia. Increased serum leptin may stimulate the leptin-LR pathway on endothelial cells to recruit inflammatory cells in IHCA. To study this hypothesis, we investigated leptin exposure on two endothelial cell models in **chapter 4**, in this case human umbilical vein endothelial cells and hepatic sinusoidal endothelial cells. Gene and protein expressions of LR and several inflammation associated factors were studied as well as leukocyte-endothelial adhesion induced by leptin.

In **chapter 5**, we investigated the nature of the ductular cells in IHCA. We hypothesized that the ductules represent DR and are part of a regenerative activity following the IHCA related inflammatory process. In this chapter, we studied the clonal background of the tumor with emphasis on tumor cells located at the peri-PPT areas which is the location of the ductular cells. This approach was chosen

based on a parallel with the situation in the normal liver in which hepatocytes are replenished from the periportal areas (the stem cell niche). Clonal analysis of the peri-PPT areas was performed based on a mitochondrial DNA mutation assay and we applied several hepatic progenitor markers in IHCA to identify and locate the cells expressing these markers.

Finally, in **chapter 6**, the previous chapters are summarized and discussed, and perspectives for future investigation outlined.

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CHAPTER 2

How normal is the liver in which the Inflammatory Type Hepatocellular Adenoma develops ?

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ABSTRACT

The inflammatory type hepatocellular adenoma (IHCA) is a subtype of HCA which is a benign liver tumor, predominantly occurring in young women in an otherwise normal liver. IHCA contains either a mutation of gp130 or STAT3. Both mutations lead to a similar morphologic phenotype and to increased expression of C-reactive protein (CRP) and/or serum amyloid A (SAA). IHCA comprised about 40% of all HCA and is associated with obesity.

We investigated the histomorphological and immunophenotypical changes of the non-tumorous liver of 32 resected IHCA specimens. Similar types of changes are present in samples taken adjacent to tumor and distant ones. The lobular architecture is well-preserved. Mild/moderate steatosis is found in a high frequency which is in accordance with the median BMI of 32 in our cases. Of note are the regular findings of sinusoidal dilatation, single arteries and minute CRP foci which are all features of HCA. These distinct CRP foci are mostly found in cases of multiple IHCA which indicates that the remnant liver may also contain IHCA foci. These findings show that the non-lesional liver in IHCA does contain abnormalities and this may have consequences for the follow-up, especially since it is known that obesity may stimulate malignant growth.

INTRODUCTION

Hepatocellular adenoma (HCA) is a benign primary hepatocellular tumor, occurring predominantly in females in their reproductive age and is associated with long term use of oral contraceptives (1, 2). Recently, a rising incidence has been reported, partly due to improved application of diagnostic imaging techniques, e.g. CT, MRI (3). HCA is divided into 3 subgroups according to 3 different genetic mutations: hepatocellular nuclear factor-1α (HNF1α) gene mutated type HCA, β-catenin gene mutated type HCA, and inflammatory type HCA (IHCA) which contains a somatic mutation of IL6ST gene. The latter mutation, encoding gp130, is found in 60% of IHCA and a somatic mutation of STAT3 gene is found in 12% of IHCA (4, 5). A fourth group represents HCA without any of these mutations. Of note, the IHCA may concurrently contain β-catenin mutation which increases the risk of malignant transformation. The HCA subtyping can be performed by visualizing the coded proteins of the mutated genes by immunohistology (6-8). HCA containing HNF1α mutation shows absence of liver fatty acid binding protein (LFABP) in contrast with the diffuse hepatocytic expression of this protein in normal livers. IHCA, both those with IL6ST mutation and STAT3 gene mutation show increased C-reactive protein (CRP) and/or serum amyloid A (SAA) expression (5). HCA containing β-catenin mutation show nuclear translocation of β-catenin expression but this finding may be focal and patchy whereas an aberrant diffuse expression of glutamine synthetase (GS) is also indicative of β-catenin mutation (6-8).

IHCA represents the largest subgroup of HCA and has been reported to be related with systemic disorders, such as obesity, metabolic syndrome and alcohol abuse (7, 9). One report mentioned that IHCA patients with a high body mass index (BMI ≥25) represent 60% of their study group in which the mean BMI is 28 (9). Subgroups of HCA except the β-catenin gene mutated type rarely show malignant transformation into hepatocellular carcinoma (HCC), although a recent study reported an increased risk in HCA occurring in overweight or obese male patients (10). These findings suggest that in obese individuals the whole hepatic microenvironment is influenced by systemic factors that may favor tumor

development, in accordance with the postulation that obesity increases the risk of cancer development (11).

In the present study we investigated the histological features of the non-tumorous liver parts of 32 resected IHCA specimens, to gain insight in the hepatic microenvironment in which IHCA develops, also because IHCA are often multifocal. Therefore, knowledge about the non-lesional liver tissue that corresponds to the remnant liver after tumor resection may influence the follow-up management of IHCA patients.

We found that although the lobular architecture is largely well-preserved, the non-tumorous liver frequently shares several abnormal features with the adenoma, such as sinusoidal dilatation and single arteries. Moreover, many cases also contain several foci of minute HCA-like areas with focal increase of CRP and/or SAA. These findings suggest that the non-lesional part of HCA-containing livers harbor changes that may potentially stimulate adenomatous growth. This is especially true for livers with multiple adenomas.

PATIENTS AND METHODS

Patients

Thirty two patients, all of them females (mean age 33.5 ± 8.8 years), who underwent partial liver resection for IHCA were included. Cases were selected on the availability of sufficient amount of adjacent non-tumorous liver (AL) and/or distant non-lesional liver tissue (DL). The latter sample was taken at least 3 cm distant from the tumor.

Histology

A representative slide of the transformation area of tumor and adjacent non-tumorous liver tissue (AL, n=32) and one sample from the distant non-lesional part (DL, n=22) were reviewed without knowledge of clinical data and the features of the corresponding tumor. Slides were stained with hematoxylin-eosin (HE) and

Masson trichrome. The AL and DL samples were assessed separately for the following features: liver architecture, steatosis, steatohepatitis, sinusoidal dilation, single artery, ductular reaction (DR). Grading of steatosis and steatohepatitis was performed according to the scoring system for Non-Alcoholic Steatohepatitis (NASH) proposed by Brunt et al (12). In summary, steatosis: 0 = absent; 1 = steatosis observed in up to 33%; 2 = more than 33% and less than 66%; 3 = more than or equal to 66%. Steatohepatitis: 0 = absent; 1 = occasional ballooned hepatocytes, mild portal chronic inflammation; 2 = obvious ballooned hepatocytes, portal and intra-acinar chronic inflammation noted, mild to moderate; 3 = ballooning and disarray obvious with mild chronic inflammation, portal chronic inflammation mild or moderate.

Grading of sinusoidal dilation followed the criteria mentioned by Rubbia-Brandt et al (13). Sinusoidal dilation: 0 = absent; 1 = centrilobular involvement limited to one-third of lobular surface; 2 = two-thirds lobular surface involved; 3 = complete lobular surface involved. Liver architecture is scored as preserved (1) or abnormal (0). Single artery and DR are described as absent (0) or present (1). Single arteries are defined as arterial structures without accompanying bile duct and/or not localized in a portal tract structure. Assessment of DR is described below.

Immunohistochemistry

The immunohistological expression of SAA and/or CRP on tumor tissue was already performed at an earlier, diagnostic stage to establish the diagnosis of IHCA according to the Bordeaux classification (7). GS and β-catenin staining were also completed at the earlier diagnostic stage to assess possible β-catenin mutation. For the present study, AL and DL samples were stained according to the same protocol and additional immunostaining with K19, CD34 and α-SMA were performed. K19 increased the feasibility to assess DR as the ductular structures were highlighted by K19 labeling. The presence of 4 or more ductular profiles per portal tract is regarded as presence of DR (14).

CD34 visualized sinusoidal capillarization and single arteries whereas α-SMA

labeled myofibroblastic transformation of hepatic stellate cells. The antibodies used for the immunohistological staining are mentioned in table 1 including the applied dilutions and retrieval methods.

Table 1: Antibodies applied for immunohistology

Antibody	Dilution	Retrieval methods	Company
β-catenin	1:100	Tris-EDTA	BD Transduction (USA)
GS	1:4000	Tris-EDTA	Millipore (USA)
CRP	1:200	Tris-EDTA	Abcam (UK)
SAA	1:200	Protease 8 min	Dako (DK)
CK19	1:100	Protease 12 min	BD Bioscience (USA)
CD34	1:20	Tris-EDTA	Dako (DK)
α-SMA	1:800	Tris-EDTA	Dako (DK)

RESULTS

Architecture: generally well preserved

In all AL and DL samples the overall lobular architecture was largely well preserved. A normal distribution pattern of portal tracts and central veins was recognizable. The transition from lesional to non-lesional tissue was usually recognizable by the slightly pushing, irregular border of the non-encapsulated tumor, except in hemorrhagic or necrotic parts where a fibrous scar may have developed and form a capsule. The regular transitional areas showed smaller, compactly arranged tumor-hepatocytes to slightly larger hepatocytes of the non-lesional part, containing more cytoplasm. Portal tracts in these transitional areas frequently contained several thick walled arteries but otherwise included normal bile ducts and portal veins. Portal inflammatory infiltrates varied but was usually non-conspicuous. Fibrosis was usually absent.

Figure 1 illustrates the several aspects of the transitional area.

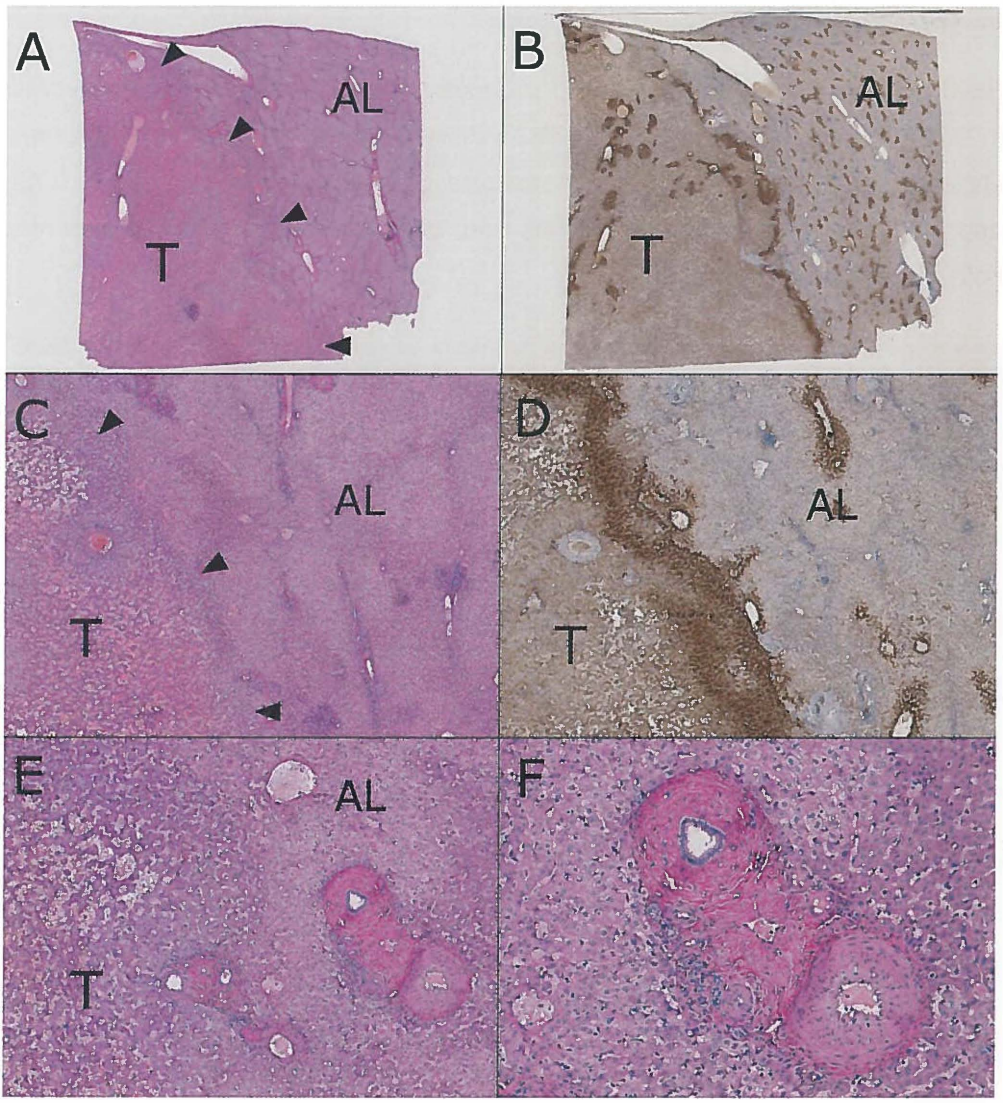

Figure 1. The transitional area of IHCA and adjacent liver

A and C: HE stained whole slide [A] and detail [C] of a transitional area of an IHCA (T) and the non-tumorous adjacent liver (AL). Arrowheads indicate the non-capsulated border of the tumor.

B and D: Glutamine synthetase expression of [A] and [C] highlights the difference in architecture of IHCA and adjacent liver (AL). In the AL part, glutamine synthetase expression in perivenular areas accentuates the preserved lobular architecture.

E and F: Portal tracts containing thick walled arteries at the border of tumor (T) and adjacent liver (AL).

Steatosis: common finding

Steatosis was a common finding in the non-lesional liver tissue as it was observed in 23/32 (70%) AL samples and 13/22 (59%) DL ones. The majority of cases showed mild to moderate degrees of steatosis. Severe steatosis is present in 3 AL and 2 DL samples. Steatohepatitis was rare, being present only in 2/32 patients, both in the AL and DL samples.

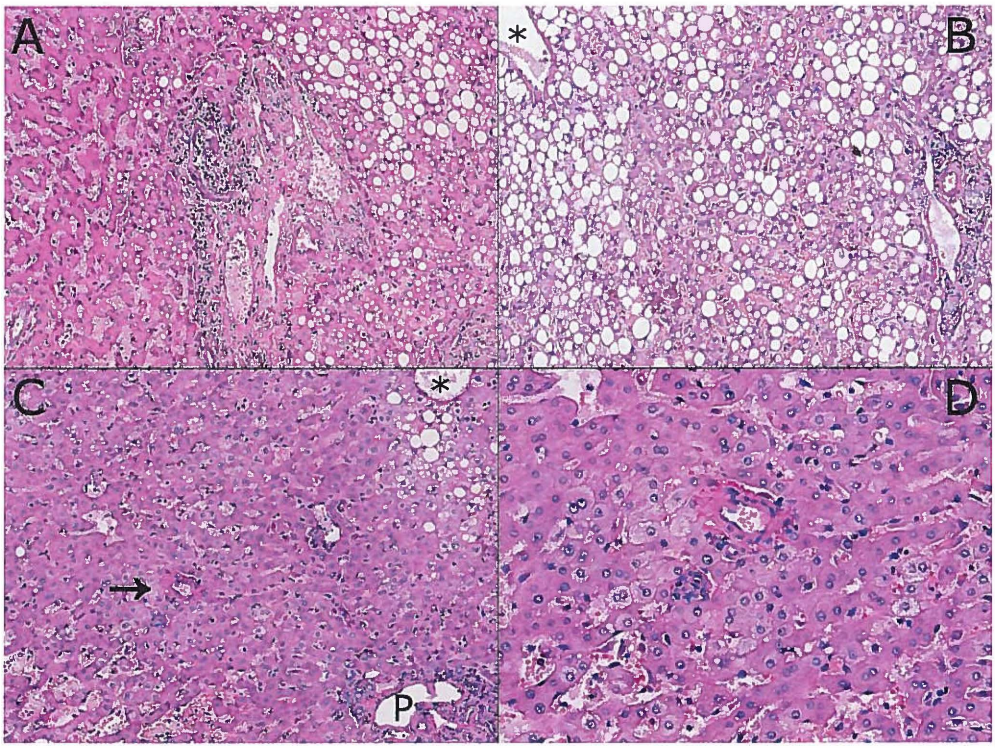

Figure 2. Steatosis and vascular changes

A: The transitional area of a non-steatotic adenoma (left part) with a steatotic adjacent non-tumorous liver (right part).

B: Moderate steatosis in a distant sample. A portal tract is present in the right lower corner and a central vein in the left upper corner (*).

C: Vascular changes in a distant sample. A portal tract (P) is present in the right lower corner and a central vein in the right upper corner (*). An area with dilated sinusoids is present in the left lower corner. The arrow indicates a group of single arteries.

D: Detail of the single arteries.

In the IHCA itself, steatosis was less common than in the non-tumorous counterpart. Steatosis was present in 15/32 (47%) tumor samples. In the steatotic liver, based on the steatosis of AL samples, there was a similar frequency of IHCA with (12/23, 52%) and without fatty changes (11/23, 48%) whereas in the non-steatotic liver most tumors were non-steatotic (6/9, 67%). A steatotic tumor in a non-steatotic liver is less common (3/9, 33%). When the frequencies were based on the steatosis of the DL samples, the majority of tumors in the steatotic liver contained fatty changes (8/13, 62%). Similar with the findings of the AL samples, tumors of non-steatotic DL samples were mostly non-steatotic as well (6/9, 67%).

Of note, 20 of the 32 patients have high BMI values, leading to a median BMI of 32.55 ± 4.9.

Figure 2 (A/B) shows the steatotic changes in the transitional area and in a DL sample.

Sinusoidal dilatation: frequent phenomenon

SD was a frequent phenomenon in both AL and DL parts showing a frequency of 59% (19/32 cases) and 77% (17/22 cases) respectively. The areas of dilated sinusoids were of variable extent and rather randomly distributed in the lobules unlike the regular centrilobular punched-out pattern of outflow-obstruction. Nevertheless, we have applied the Rubbia-Brandt scoring system (13) that follows the lobular architecture to allow a semiquantitative scoring. In both AL and DL samples the vast majority of SD was of mild degrees, as observed in 74% and 76 % of those cases showing SD. Of the 5 cases with moderate and severe SD in their AL samples, 3 cases showed mild SD in their corresponding DL, 1 case had moderate SD and 1 case had similarly severe SD in their DL. The 2 latter cases represented 2 of 4 DL cases with moderate and severe SD. The 2 remaining DL cases showed both mild SD in their corresponding AL.

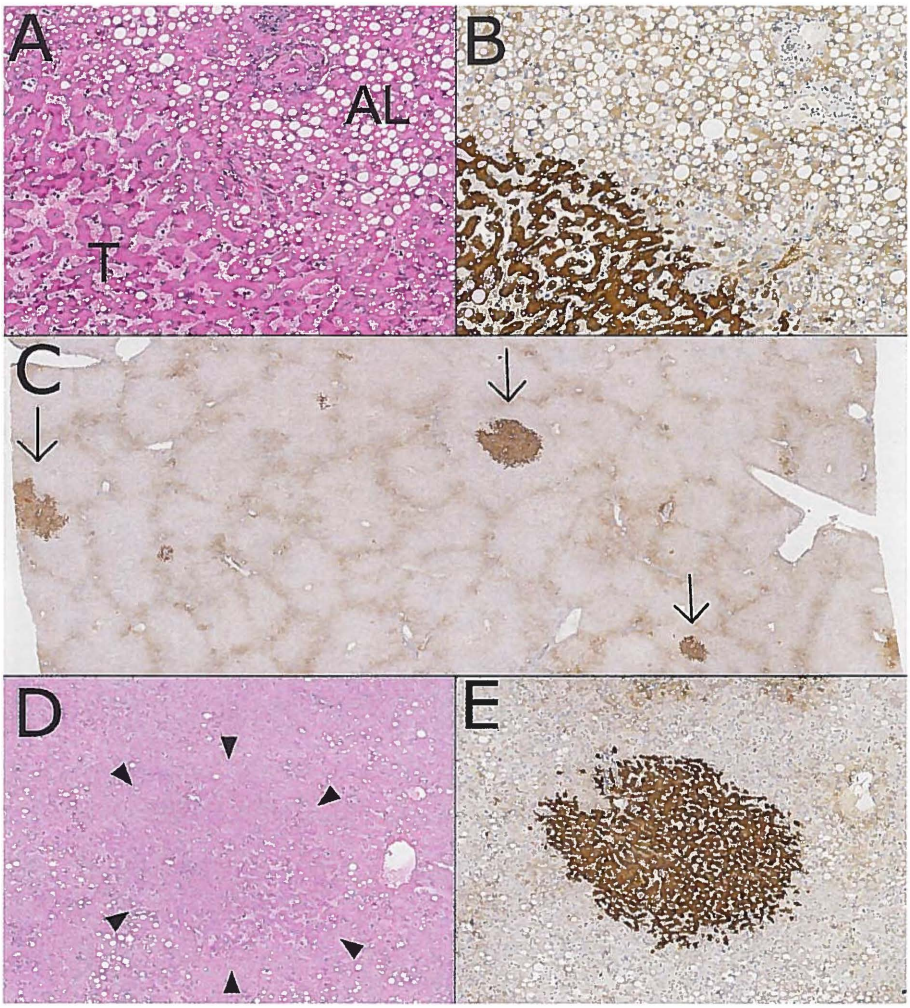

Figure 3. CRP expression in the transitional area and minute CRP positive foci in a distant sample.

A: The transitional zone of an adenoma without steatosis (T) and steatotic adjacent liver (AL) in hematoxylin-eosin (HE) staining.

B: The same area as [A] in CRP immunostaining showing diffuse increase of CRP in the tumor part.

C: CRP immunostaining of a non-tumorous liver sample distant from the tumor showing 3 minute CRP foci (arrows). The preserved architecture of the hexagonal liver lobules is highlighted by the vague expression of CRP which outlines the peripheral boundaries of the lobules.

D: Detail of a minute CRP positive focus in HE showing an area in the lobule with slightly dilated sinusoids, more eosinophilic hepatocytes and absence of steatosis which is present outside the contours of this focus (arrowheads).

E: The CRP expression of the focus described in D.

Single arteries/arterioles: regularly seen, even in distant samples

There was a similar frequency of single arteries/arterioles in AL and DL samples. Single arteries were present in 12/32 AL samples (38%) and in 8/22 DL samples (36%). As in the HCA, these single arteries were both present in small groups and as truly single arterial structures in the hepatic lobule (Figure 2 C/D).

Immunohistology: minute foci of CRP positive areas; ubiquitous DR and activated myofibroblasts

The expression pattern of GS confirmed the preserved lobular architecture in AL and DL samples as shown by the perivenular distribution of cytoplasmic GS in hepatocytes in the centrilobular areas (Figure 1 A-D). Bile ductal and ductular cholangiocytes showed a faint blush of cytoplasmic GS expression. A normal membranous β-catenin labeling was present in all hepatocytes but no nuclear expression. Bile ducts and ductules also showed membranous but no nuclear β-catenin expression.

All AL and DL samples showed a normal periportal pattern of CRP expression in hepatocytes. However, in 14/32 cases minute foci of aberrant CRP expression were observed in the hepatic lobule, consisting of 6 AL samples, 4 DL samples and 4 other cases of which both the AL and DL samples contained CRP positive foci. Eleven of these 14 cases concerned livers with multiple adenomas (Figure 3). In the studied group 21/32 IHCA were multiple adenomas. None of the minute CRP positive foci showed GS positivity and/or nuclear β-catenin expression.

Additional immunohistology was performed with K19, CD34 and α-SMA. DR was practically ubiquitous, being present in 29/32 (91%) AL samples and 21/22 DL (95%) ones (Figure 4 A).

CD34 staining showed a normal distribution pattern of vascular endothelial labeling and periportal sinusoids. There was no increase of CD34 expression in the rest of the sinusoids. In contrast with CD34, an increased α-SMA expression in the sinusoids was seen in 28/32 (88%) AL and 17/22 DL (77%) (Figure 4 B). Diffuse increase of α-SMA was seen in 15/28 (54%) of the α-SMA positive AL samples and a focal increase localized in the areas of SD was present in 46%. A similar

frequency was observed in the DL samples with 9/17 (53%) diffuse distribution and focal increase of α -SMA in 47% of the α -SMA positive DL samples.

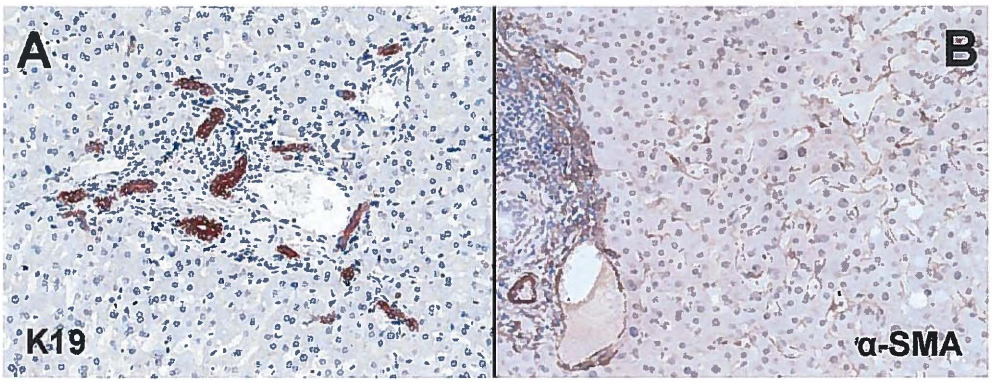

Figure 4. Ductular reaction and activated myofibroblasts in the non-tumorous liver.
A: Ductular reaction in a portal tract of a distant non-tumorous liver sample highlighted by K19 immunostaining.
B: Presence of α-SMA positive myofibroblasts in a distant non-tumorous liver sample, most obvious in dilated sinusoids (center and left lower corner).

DISCUSSION

In contrast with HCC which usually develops in a liver with long standing chronic liver disease, HCA is mostly found in an otherwise normal liver. IHCA is one of the variants of HCA representing 40-50% of all HCA (1). The 2 different mutational backgrounds of IHCA concerning gp130 and STAT3, lead to a similar morphology and immunophenotype of increased SAA and/or CRP in the tumor hepatocytes (5).

In the present study we analyzed the histological and immunophenotypical changes of the non-lesional liver parts of resected IHCA specimens which include samples taken adjacent to the tumor and distant ones. Similar types of histological and immunophenotypical features were found in these two sample types, albeit in variable degrees and frequencies. Among these changes some features represent changes that are also present in IHCA but otherwise not found in normal livers, e.g. sinusoidal dilatation, single arteries and foci of CRP positive hepatocytes.

The lobular architecture is generally well preserved as also confirmed by the normal perivenular distribution pattern of GS expression. Steatosis is very common, being present in 60-70% of the distant and adjacent non-lesional samples, which is in accordance with the high BMI of our study population and in line with the reported relation of IHCA with obesity (7, 9). Although the latter condition is known to enhance carcinogenesis (11), the tumorigenic role of obesity in (I)HCA has yet to be elucidated. In the steatotic livers, based on the pattern of the distant samples, almost two-third of the tumors contains fatty changes. In the non-steatotic livers two-third of the tumors is non-steatotic. Although much higher numbers of patients are necessary for robust conclusions, the above findings indicate that fatty changes in the tumor might be secondary to the fatty constitution of the liver in which the IHCA develops. The scarcity of steatotic tumors in non-steatotic livers supports this view.

Of note are the vascular abnormalities consisting of sinusoidal dilatation and single arteries. Several types of vascular changes in the non-tumorous liver have been described in an early study on telangiectatic focal nodular hyperplasia which is the obsolete term of IHCA according to the new classification (15). These features are not found in normal livers, neither do these features belong to the spectrum of changes of fatty liver disease, but these features are characteristics of IHCA. Single arteries are also frequently found in HCC and are even part of the criteria to establish the diagnosis of early HCC (16). Sinusoidal dilatation appeared to be a common finding in the non-lesional liver parts, both those adjacent to the tumor and the distant samples. The fact that sinusoidal dilatation is also present in the non-lesional tissue indicates a systemic effect. In our study group of women in their reproductive stage, long term use of oral contraceptives may have a contributory role as these agents are known to cause sinusoidal dilatation and peliotic changes. However, the concurrent presence of sinusoidal dilatation and single arteries in the non-lesional liver parts is suggestive for a common background factor leading to these vascular abnormalities. In HCA, these changes have been related to an increased gene expression of Angiopoietin-1, a vascular growth factor of the Angiopoietin/Tie-2 system (17, 18). Excess Ang-1 has been reported, both in animal models and *in vitro* to induce vascular remodeling

including dilated sinusoids and vessel forming capacity (19-21). In another study we have found increased Ang-1 in HCC (22) in which single arteries are frequently found. These arteries increase in numbers paralleling tumor growth in HCC and this phenomenon is regarded as tumor angiogenesis as the arteries form the vascular supply of the growing tumor (23). The fact that there is no obvious tumor growth in the non-lesional liver of our IHCA study group renders angiogenic activity in these parts rather redundant. It is however plausible that an excess of Ang-1, produced by the tumor, may exert its effects in the non-lesional parts. In particular, because Tie-2 receptor which is the specific tyrosine kinase receptor of Ang-1, is ubiquitously present on the sinusoidal endothelial cells and vascular endothelial cells of histologically normal livers (17).

The dilated sinusoids usually lead to variable degrees of atrophy of the hepatic parenchyma. Paralleling these degenerative changes is the increased expression of α-SMA in these areas, reflecting activation of hepatic stellate cells into myofibroblasts. The latter process is probably also induced by steatosis which is present in the majority of cases and which is known to be a potent inducer of myofibroblastic activation. Variable types of hepatocellular damage are apparently present in the non-lesional liver of IHCA which would require replenishment of cellular loss. The presence of DR in nearly all samples reflects the regenerative activity. DR has been described in fatty liver disease (24) but in general it reflects a reparative activity that includes several progenitor cell niches (14).

Apart from the degenerative changes, the findings of CRP positive foci outside the tumor and within liver tissue with preserved lobular architecture is most intriguing. It is tempting to speculate that those foci may represent minute HCA, particularly because most of those foci were found in cases with multiple adenomas. Our findings largely confirm the results Bioulac-Sage et al. who also found additional CRP positive micronodules in multiple IHCA, measuring between 2 to 10 mm, and containing features of IHCA (25). These micronodules are mostly slightly larger than the CRP foci in the present study which are mostly smaller than 2 mm and in the majority of cases were found in random samples. Due to its subtlety these foci are easily overlooked, first during gross examination of the resected specimen and secondly in routine HE staining. This may probably lead to

the reported absence of these foci in many other cases of multiple IHCA. To avoid this sampling error it is recommendable to investigate the non-lesional liver tissue more robustly, e.g. include more sampling and application of additional CRP staining. If positive foci are found it may indicate the presence of minute foci of HCA in the remnant liver which may have consequences for the follow-up management. The long term behavior of small HCA foci and under specific circumstances such as pregnancy is not fully established. A recent study on the management of HCA during pregnancy has shown that discouragement of pregnancy in certain cases is no longer necessary because close monitoring of patients with small adenomas seems to offer adequate surveillance (26). Whether this applies to multiple adenomas with multiple CRP positive foci is yet unclear.

In conclusion, from the architectural point of view the non-lesional liver part of IHCA may be considered normal. However, the CRP positive foci indicate that in cases with multiple adenomas, minute foci of adenomas may be present, also in the remnant liver. The presence of vascular abnormalities beyond the tumor and beyond the CRP foci needs further study, especially due to the similarities with the changes in the HCA. The high incidence of steatosis does not only confirm the hepatic manifestation of obesity in this group of patients. It provides another evidence that the normal liver in IHCA does contain abnormalities and in selected cases should probably be considered as a diseased liver.

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CHAPTER 3

Increased Tumor Leptin-Receptor: Linking Obesity With The Inflammatory Type Hepatocellular Adenoma

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ABSTRACT

Inflammatory type hepatocellular adenoma (IHCA), one of the 4 variants of HCA, is a benign primary hepatic neoplasm which is associated with obesity. The pathogenetic mechanism behind this association is largely unknown. We investigated the tissue expression profile of leptin, adiponectin, their receptors and downstream signalling factors in IHCA samples of 25 patients whose median body-mass-index was 30.1.

Leptin and adiponectin mRNA was not detectable in IHCA, non-lesional livers and normal livers, using real time RT-PCR and immunohistochemistry, whereas ample quantities were found in control adipose tissue. Increased gene expression of the leptin receptor (LR) was found in tumor tissue compared to the non-lesional liver, whereas AdiR2, the liver relevant adiponectin receptor-2 was decreased. Both receptors were mainly expressed by sinusoidal and vascular endothelial cells (EC). Signal transducer and activator of transcription-3 (STAT3) gene expression, the downstream intracellular factor of LR signalling, was higher in the tumor tissue than in non-lesional tissue, and nuclear expression of phosphorylated-STAT3 was visible in tumor and non-lesional liver. In a complementary *in vitro* study, EC showed increased expression of LR and interleukin-6 upon stimulation by leptin.

Conclusion: LR expression and activation of the leptin/LR pathway are increased in IHCA, probably due to obesity related hyperleptinemia which can induce LR expression on tumor EC. Increased leptin/LR activity may contribute to the growth of IHCA based on the established role of leptin as promoter of proliferation and angiogenesis. These findings provide a first pathogenetic link in the association between IHCA and obesity.

INTRODUCTION

Hepatocellular adenoma (HCA) is a benign hepatocellular neoplasm, predominantly found in women in their reproductive stage. The current classification of HCA is based on several mutational changes in the different subtypes (1, 2) and the subsequent aberrant expression of several proteins that can be visualized by immunohistology, by which method the HCA subtypes can be determined on the tissue level (2, 3).

Among the 3 known subtypes, the inflammatory type HCA (IHCA) is a common variant with frequencies ranging from 30-50% based on examination of resection specimens (1, 4, 5). The distinctive immunophenotype of IHCA is characterized by overexpression of the acute phase proteins C-reactive protein (CRP) and/or serum amyloid A (SAA) protein in the tumor cells. This immunophenotype is present in all IHCA regardless of its mutational background (6). In 60% of IHCA a somatic mutation of interleukin-6 signal transducer (IL6ST) is encountered that encodes gp130, a co-receptor and signal transducer of interleukin-6 (IL-6), leading to activation of signal transducer and activator of transcription-3 (STAT3) (7). More recently, a somatic STAT3 mutation was found in 8-12% of IHCA cases without an IL6ST mutation (8). However, there were no significant differences in the expression of the target genes of IL-6 and interferon (IFN) pathways between these 2 types of mutations (8).

A pivotal clinical feature of patients with IHCA is the frequent association with an increased body mass index (BMI) (6, 9, 10). One study reported a mean BMI of 28 and the presence of 60% overweight patients in the studied population (9). The same group of investigators has corroborated the significance of obesity in patients with HCA by documenting a significantly increased frequency of malignant transformation of HCA into hepatocellular carcinoma (HCC) in male patients with metabolic syndrome (11). This finding is in line with the growing body of evidence supporting the positive link between obesity and an increased risk of several types of cancer, including HCC (12-15). In overweight individuals there is alteration of the physiologic functions of adipose tissue and expansion of adipose tissue depots, including the visceral adipose tissue (VAT). VAT has been recognized as being

metabolically more active than subcutaneous adipose tissue and is an important player in hepatic diseases. The portal circulation enables direct targeting of the liver by factors released by VAT, including adipokines (16). The dysfunctional adipose tissue also stimulates an inflammatory state via increased release of IL-6 and tumor necrosis factor-α (TNF-α), and via reduction of the anti-inflammatory effects of adiponectin (17).

Of the 2 most known adipokines, leptin and adiponectin, it is furthermore recognized that leptin is not only a proinflammatory factor but it also stimulates angiogenesis and hepatocyte proliferation both in liver regeneration and malignant growth in HCC (16, 18).

The increased level of serum CRP in IHCA patients and their high BMI indicate an inflammatory state that is probably associated with obesity but the pathogenetic mechanisms underlying these associations are as yet unexplored.
We hypothesized that in IHCA patients, the postulated increase of serum leptin and decreased levels of adiponectin in overweight individuals may be associated with altered profiles of adipokines at the tumor tissue level which may favor tumor growth and increase a pro-inflammatory phenotype.

Therefore, we investigated the gene expression and cellular localization of leptin, adiponectin, their receptors and downstream signaling factors of leptin activation in samples of IHCA obtained from 25 patients.

We studied the leptin receptor (LR) encoded by the LEPR gene and adiponectin receptor 2 (AdiR2), encoded by the ADIPOR2 gene as it is the most relevant type of adiponectin receptor in the liver (16, 19). There was a significantly higher gene expression level of LR and lower level of AdiR2 in the tumor as compared with the adjacent non-tumorous tissue and normal liver samples. LR *b* form (LRb) is the functional isoform which can activate the JAK2/STAT3 pathway. Downstream activation of leptin-LRb in IHCA was shown by the presence of nuclear p-STAT3 expression in tumor cells. To investigate the interplay between leptin and LR expression we tested the effect of leptin stimulation on endothelial cells (EC) in an *in vitro* assay. EC was chosen because we have documented by immunohistology that LR is mainly expressed by sinusoidal and vascular EC in the tumor, and that it was absent in hepatocytes. The study showed that leptin-

stimulated EC showed an increased gene expression of IL-6 and LR. These findings indicate that hyperleptinemia in obese patients with IHCA may stimulate leptin-signaling on tumor EC, which will stimulate tumor growth and contribute to its inflammatory phenotype due to the established potentials of leptin as proinflammatory and growth-promoter (16).

MATERIALS AND METHODS

Patients

Archival tumor samples of 25 IHCA patients were selected based on the availability of frozen samples and paraffin sections. All patients were female with a median age of 29 year (range 19-53) and a median BMI of 30.1 (range 18.3-41.8). All tumors were immunohistologically subtyped as IHCA according to internationally accepted criteria (1-3). Thirteen samples of normal controls (median BMI 21.7) were taken from non-lesional parts of resected liver specimens containing hemangioma or focal nodular hyperplasia, and from surplus donor liver after partial liver transplantation. The non-lesional parts of the IHCA liver samples were also included in the study and these samples were harvested at least 3 cm distant from the tumor. Anonymous usage of tissue is in accordance with current local ethical guidelines.

Histology

Grading of steatosis and inflammation, and staging of fibrosis in non-tumorous liver were defined according to the scoring system (NAS) for Non-Alcoholic Steatohepatitis (NASH) proposed by Brunt et al (20, 21). Steatosis in IHCA was assessed in the most steatotic areas and graded as follows: steatosis less than 5% in the tumor parenchyma was graded as grade 0, between 5% and 33% as grade 1, between 33% and 66% as grade 2 and more than or equal to 66% as grade 3.

Quantitative RT-PCR

Frozen samples of each case were collected in RNA-free tube and preserved at -80°C. Total RNA was isolated with the RNeasy Mini Kit and RNeasy Plus Universal Kit special for fat RNA isolation, and was freed from DNA contamination with gDNA Eliminator spin column (Qiagen, Venlo, the Netherlands). All protocols were provided by the kit. RNA integrity was checked by electrophoresis and RNA quantity was analysed by Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). 1 µg of total RNA was reverse transcribed into cDNA using superscript III RT (Invitrogen, Bleiswijk, the Netherlands), according to the manufacturer's instructions. 10 ng cDNA was consumed for each following 10 µl PCR reaction. Exons overlapping primers for real-time PCR were purchased from Applied Biosystems (Nieuwekerk a/d IJssel, the Netherlands): housekeeping gene (GAPDH, assay ID Hs99999905_m1), leptin (LEP, assay ID Hs00174877_m1), leptin receptor (LEPR, assay ID Hs00174497_m1), adiponectin (ADIPOQ, assay ID Hs00605917_m1), adiponectin receptor 2 (ADIPOR2, assay ID Hs00226105_m1), STAT3 (STAT3, assay ID Hs01047580_m1), signal transducer and activator of transcription-5a (STAT5a) (STAT5A, assay ID Hs00234181_m1), signal transducer and activator of transcription-5b (STAT5b) (STAT5B, assay ID Hs00273500_m1), suppressor of cytokine signaling 3 (SOCS3) (SOCS3, assay ID Hs02330328_s1). Absolute quantitative PCR was performed in an ABI 7900HT Sequence Detector (Applied Biosystems, CA, USA) with cycling conditions of 15 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C. Cycle threshold (Ct) was defined as the cycle number at which a significant increase in the fluorescence signal was first detected. Triplicate real-time PCR was performed for each sample. Gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the relative gene expression value. Control samples consisted of Milli-Q water and occasional RNA samples were consistently found negative.

An additional analysis for leptin gene expression was performed using SYBR Green real time PCR as a parallel trial in liver and fat samples. The hLEP designed primers were purchased from Biolegio (Biolegio BV, Nijmegen, the Netherlands). The sequence of LEP forward primer was CATTTCACACACGCAGTCAGTCT (5'-3'), the reverse sequence was CAGTGTCTGGTCCATCTTGGATAA (5'-3') (22).

0.25 µl 6 µM forward primer, 0.25 µl 6 µM reverse primer, 5 µl SYBR green mastermix and 10 ng cDNA were reacted in each 10 µl PCR reaction. Each sample was analysed in triplicate and yielded Ct values after comparing with hGAPDH analysed in the equipment described above.

Immunohistochemistry

Frozen sections of 4 µm were used for immunohistochemical staining for leptin, LR, adiponectin, AdiR2, and 4 µm paraffin sections for phospho-stat3 (p-STAT3) and phospho-stat5 (p-STAT5) staining. Antibodies, dilutions and sources are summarized in Table 1. Frozen slides were air-dried at room temperature for 20 min, fixed in acetone before incubation with the primary antibody. Paraffin slides were deparaffinized and endogenous peroxidase was blocked with 0.3% H_2O_2 for 30 min. Antigen retrieval by heating in pH 8.0 ethylenediaminetetraacetic acid (EDTA) buffer in microwave for 15 min was performed for the paraffin sections. Slides were incubated overnight with the primary antibodies at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary and tertiary antibodies for 30 min each at room temperature. Labeling was visualized by application of AEC (3-amino-9thyl-carbazole, Sigma-Aldrich, St. Louis, MO, USA) or DAB (3,3'-diaminobenzidine, Sigma-Aldrich, St. Louis, MO, USA) for 15 min and hematoxylin counterstaining for 1 min.

Table 1 Antibodies used in immunohistochemistry

Antibody	Dilution	Company	Code
Leptin	1:50	Abcam	ab3583
Leptin receptor	1:10	Santa cruz	sc-1834
Adiponectin	1:25	Abcam	ab22554
Adiponectin receptor 2 (AdiR2)	1:25	Abcam	ab53399
Phospho-stat3 (p-STAT3)	1:100	Cell Signaling	#9145
Phospho-stat5 (p-STAT5)	1:25	Cell Signaling	#9359
Envision+System-HRP(AEC)	Ready to use	Dako	K4009
HRP-conjugated goat anti rabbit Ig	1:100	Dako	P0448
HRP-conjugated rabbit anti goat Ig	1:100	Dako	P0160
HRP-conjugated rabbit anti mouse Ig	1:100	Dako	P0260

Laser microdissection (LMD) and isolation of RNA

Since we failed to detect LR protein expression on hepatocytes in immunohistochemistry, we decided to measure the LR gene expression in hepatocytes collected by LMD. We harvested clusters of hepatocytes from 2 IHCA samples while avoiding pseudo portal tracts and obvious veins (Figure 7 A). CD34 gene expression (which is expressed by sinusoidal EC in IHCA) analysis was also applied to check the purity of the hepatocyte samples after LMD.

Nine micron frozen sections of two tumor samples were cut on the 1.0 PEN membrane slides (Carl Zeiss Microscopy GmbH, Göttingen, Germany) after UV irradiation and stained with hematoxylin-eosin. After overnight drying the sections, clusters of hepatocytes (Figure 7 A) were laser microdissected and collected in adhesive caps until reaching 1.2 million μm^2 and preserved at -80°C. Total RNA of laser dissected samples was isolated with RNeasy Micro Kit by instructions (Qiagen, Venlo, the Netherlands). cDNA synthesis of these samples was performed as described above. Housekeeping gene GAPDH, LEPR, and CD34 (assay ID Hs00990732_m1) were applied by real-time PCR. All PCR reactions were performed in duplicates. Gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the relative gene expression value.

Western blot

Frozen samples of 3 pairs of IHCA including tumor and corresponding distant liver and 2 normal liver samples were collected for western blot. Ten sections of ten micron thick were collected from each frozen sample which minimally measured 1cm×1cm, and lysed in 100 µl radio immuno precipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate and 0.1% SDS) containing protease inhibitor cocktail (Roche Diagnostics, Almere, the Netherlands). After measuring the protein concentration by DC protein Assay following the manual (BIO-RAD, Veenendaal, the Netherlands), 30 µg protein of each sample was separated on 7% SDS-PAGE for LR protein detection and transferred to nitrocellulose membranes (GE Healthcare Europe GmbH, Diegem, Belgium). Membranes were blocked with 5% non-fat dry milk (Campina, Woerden, the Netherlands) in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST)

for 1.5 hours, and then probed with the following primary antibodies: rabbit anti-LR polyclonal antibody (Santa Cruz, Bioconnect, Huissen, the Netherlands) diluted 1:400 in 5% non-fat dry milk/TBST, monoclonal mouse anti-β-actin (Abcam, ITK Diagnostics, Uithoorn, the Netherlands) diluted 1:4,000 in 5% non-fat dry milk/TBST. The horseradish peroxidase-conjugated secondary goat anti-rabbit and rabbit anti-mouse antibodies (Dako, Glostrup, Denmark) used for detection were diluted 1:2,000 in 5% non-fat dry milk/TBST and visualized using the Pierce ECL2 chemoluminoscence detection kit (Thermo Scientific, Rockford, USA).

Endothelial cell activation with leptin

Human umbilical vein endothelial cells (HUVEC, Lonza Walkersville Inc. Walkersville, USA) and human hepatic sinusoidal endothelial cells (hHSEC, ScienCell Research Laboratories, San Diego, USA) were studied. HUVEC were grown in EGM2-MV (CC 3156, Lonza Walkersville Inc) with 5% fetal calf serum (FCS), penicillin, and endothelial cell growth factor. hHSEC were cultured in ECM (No. 1001, ScienCell Research Laboratories) with 5% FCS, penicillin/streptomycin solution, and endothelial cell growth supplement. Human recombinant leptin (R&D Systems, Abingdon, UK) was reconstituted at 1mg/ml within sterile 20mM Tris-HCL pH 8.0.

HUVEC and hHSEC harvested between the fourth to sixth passages were seeded in 24-well plates at 70%-80% confluence 1 day prior to starvation with 1% FCS for 16 hours. Then they were exposed to control medium (1%FCS), vehicle (2% Tris-HCL) and 200 ng/ml leptin for 12 hours and 24 hours. Total cellular RNA was isolated with RNeasy Mini Kit, reversed transcribed into cDNA and quantitative PCR studies were performed according to the methods described above. Primers for the housekeeping gene, LR and IL-6 (assay ID Hs00174131_m1) were same with described above. Gene expression was compared to control and expressed as mean fold change ± standard deviation (SD).

Statistics

Data were analysed by PASW Statistics 18 software (SPSS, Chicago, IL, USA) and presented as median values and interquartile range. Data with a normal distribution were analysed with Student's t-test for analysis between two groups, and paired t-test when comparing paired samples. Non-parametric tests were applied when analysing data without a normal distribution. One-way ANOVA with Bonferroni correction was performed in comparing means among more than two groups. The relationship between discrete variables was evaluated with chi-square test. $p<0.05$ was set as the level of significance.

RESULTS

Histology:

11/25 of IHCA and 12/25 of the non-tumorous liver showed steatosis

All IHCA cases fulfilled the immunophenotypic criteria of diffuse increase of CRP and /or SAA (Figure 1 A, B). Steatosis in the tumor was seen in 11 of 25 cases (44%), the majority being of grade 1-2 (8 out of 11 cases) and 3 cases of grade 3. Steatosis in both tumor and non-tumorous liver was present in 6 cases. In 5 cases only the tumor was steatotic and in 6 cases only the non-tumorous liver was steatotic.

In the non-tumorous livers steatosis was observed in 48% (12/25) of cases; grade 1-2 in 9/12 cases and 3 cases of grade 3 (Figure 1 C, D). Features compatible with NASH, consisting of steatosis, hepatocytic ballooning and lobular inflammation were present in 10 out of these 12 cases; 5 cases with NAS≥5 and 5 cases with 3<NAS<5.

There is no correlation between BMI and steatosis neither in tumor nor in non-tumorous liver of IHCA.

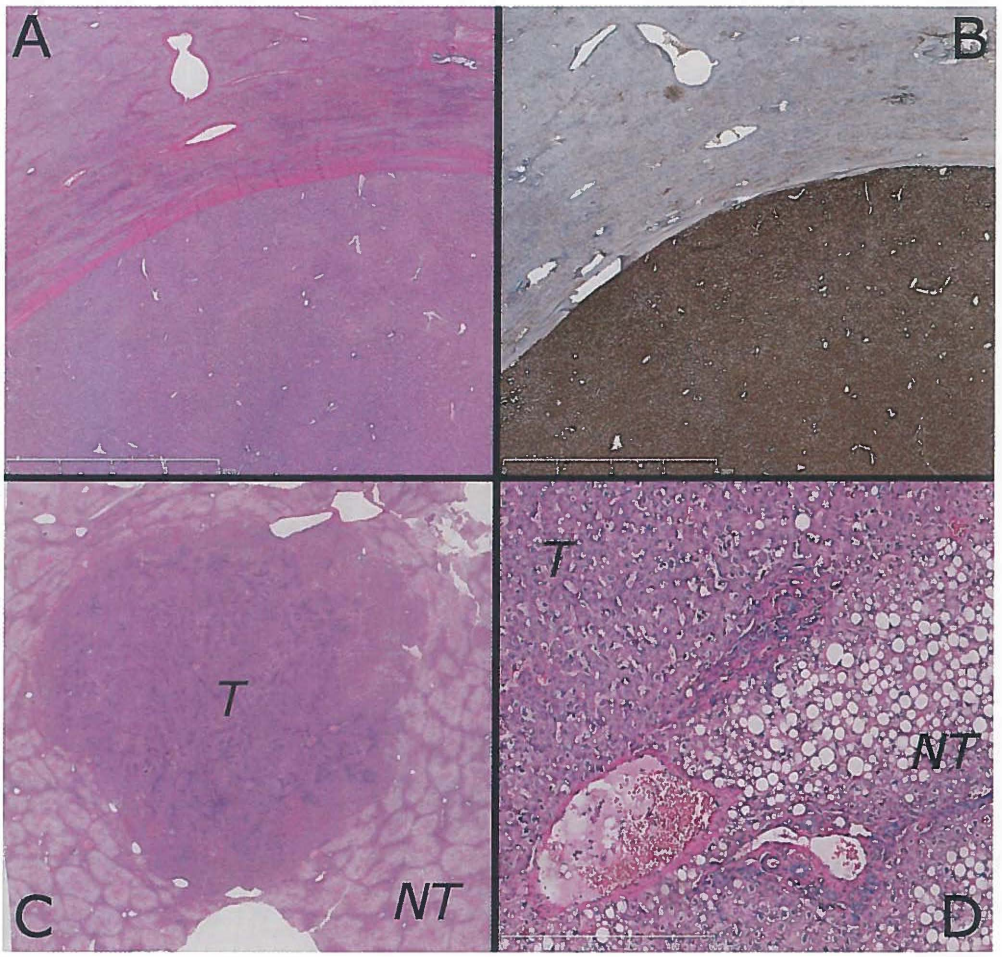

Figure 1. Histology of the inflammatory type hepatocellular adenoma (IHCA)

A: HE staining of the transformation zone of tumor (lower half) and non-lesional liver (upper half).

B: The same area as in "**A**" showing diffuse increased expression of C-reactive protein staining in the tumor.

C: steatotic adjacent liver (*NT*) with non-steatotic tumor (*T*) (5 ×);

D: higher magnification of the margin of steatotic adjacent liver (*NT*) with non-steatotic tumor (*T*) (100 ×).

Gene Expression

1. Higher LR and lower AdiR2 in tumor than adjacent liver and normal liver

LR gene expression was significantly up-regulated in the tumor compared to non-tumorous adjacent liver (p=0.021) and to normal liver (p= 0.026) (Figure 2). AdiR2

gene expression was significantly lower in the tumor compared with the adjacent liver (p=0.014), while there was no significant difference with normal livers.

These findings were confirmed when paired tumor samples and their non-tumorous counterparts (16 cases) were analysed separately. Gene expression of LR was significantly higher (p=0.021) whereas AdiR2 was lower in the tumor than the non-tumorous liver (p=0.021) (Figure 3).

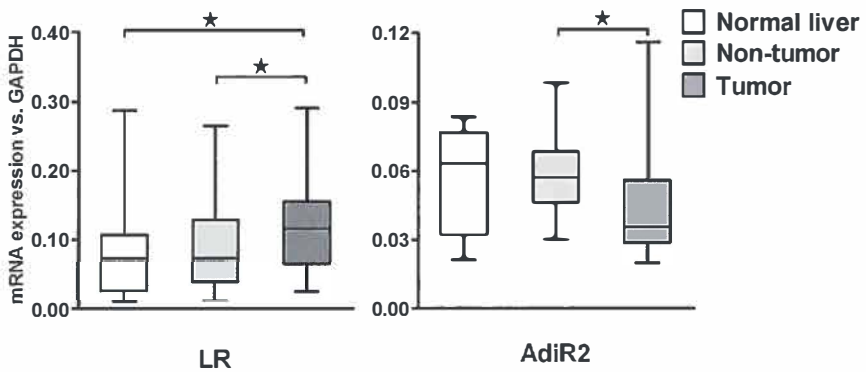

Figure 2. Leptin receptor (LR) and Adiponectin receptor 2 (AdiR2) relative gene expression in tumor, non-tumor and normal liver. mRNA levels shown are relative to GAPDH and detected by quantitative RT-PCR as described in MATERIALS AND METHODS.

White box, normal liver (n=13); light gray box, non-tumor (n=19); dark gray box, tumor (n=22). ★ = p<0.05.

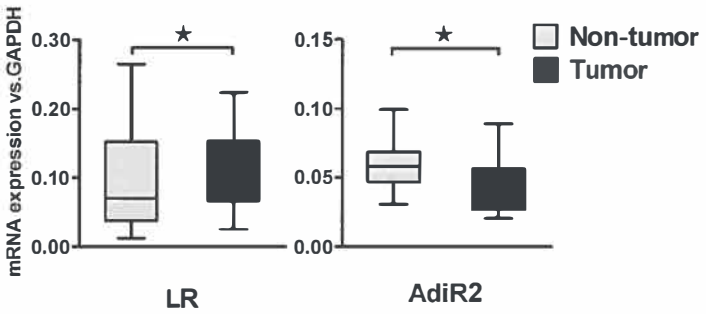

Figure 3. Leptin receptor (LR) and adiponectin receptor 2 (AdiR2) relative gene expression in paired samples. mRNA levels shown are relative to GAPDH and detected by quantitative RT-PCR as described in MATERIALS AND METHODS.

Light gray box, non-tumor (n=16); dark gray box, tumor (n=16). ★ = p<0.05.

Effect of BMI: tumor LR higher in patients with BMI < 30

When the results were regrouped according to the patients' BMI status, IHCA of patients with BMI < 30 showed significantly higher LR gene expression as compared to normal controls (p=0.032). This difference was not observed between the IHCA of patients with BMI ≥30 and the normal control samples (Figure 4).

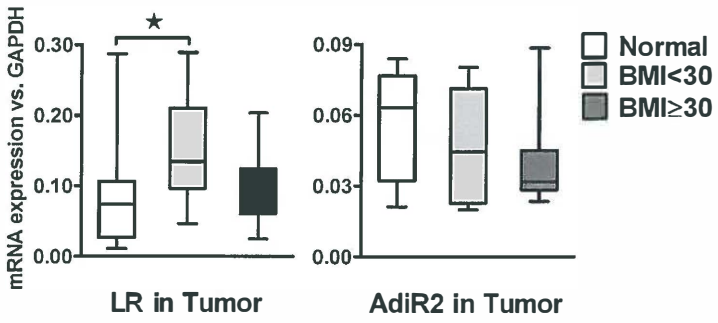

Figure 4. Leptin receptor (LR) and adiponectin receptor 2 (AdiR2) relative gene expression in BMI≥30 or <30 tumors. mRNA levels shown are relative to GAPDH and detected by quantitative RT-PCR as described in MATERIALS AND METHODS.

White box, normal liver (n=13); light gray box, tumor with BMI<30 (n=8); dark gray box, tumor with BMI ≥ 30 (n=11). ★ = p<0.05.

Effect of steatosis in the tumor: higher LR in low grade steatotic tumors

Tumors containing low grade steatosis (grade 0-1) showed a higher LR gene expression level (p=0.031) compared to normal control livers whereas tumors with high grade steatosis did not show a significant difference with normal livers. When tumors were grouped according to the degree of steatosis of the non-lesional livers there were no significant differences in gene expression levels. There were also no differences of tumor LR gene expression between cases with or without NASH (data not shown).

2. Leptin and Adiponectin not detectable in liver

There is no hepatic mRNA expression of leptin and adiponectin in tumor, non-tumorous liver and normal liver samples, while both genes were amply detectable in adipose tissue samples that were used as positive controls. Similar results were

found in the parallel measurement for LEP gene expression with SYBR green assay (data not shown).

3. Higher STAT3 expression in tumor

Gene expression of STAT3, STAT5a, STAT5b and SOCS3 were measured to test the downstream effects of activation of the leptin receptor pathway. There is a statistical borderline difference (p=0.0547) of higher STAT3 expression in the tumor compared to the adjacent non-tumorous liver (Figure 5). STAT5a, STAT5b and SOCS3 did not show significant differences.

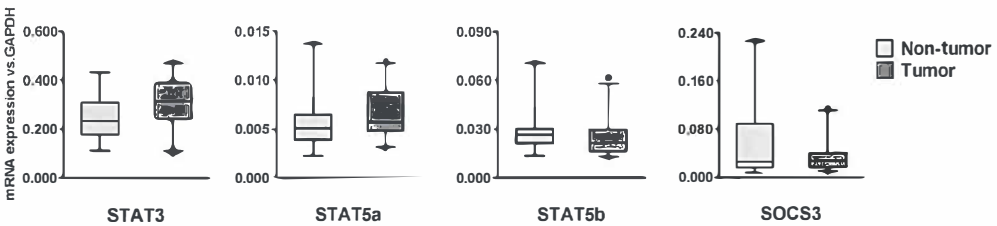

Figure 5. mRNA expression levels in tumor and non-tumorous groups of several downstream signaling factors of the leptin-leptin receptor pathway. mRNA levels shown are relative to GAPDH and detected by quantitative RT-PCR as described in MATERIALS AND METHODS.
Light gray box, non-tumor (n=19); dark gray box, tumor (n=22).

Immunohistology

Both leptin and adiponectin were expressed by Kupffer cells and were not observed in other liver cell constituents. LR and AdiR2 were present on sinusoidal endothelial cells (SEC), vascular endothelial cells (VEC), hepatic stellate cells (HSC), cholangiocytes of bile duct and less obvious in ductules, and also in the vessel wall. The pattern of expression was similar in tumor and non-tumorous liver tissue. (Figure 6 A, B)

Most cases showed nuclear p-STAT3 expression both in tumor and non-tumorous liver tissue in hepatocytes, SEC and VEC (Figure 6 C, D). Absence of p-STAT3 expression was only seen in 3 cases. Hepatocytic nuclear p-STAT5 expression was not seen in any tumor, while it was present in 3 cases of adjacent liver tissue (data not shown).

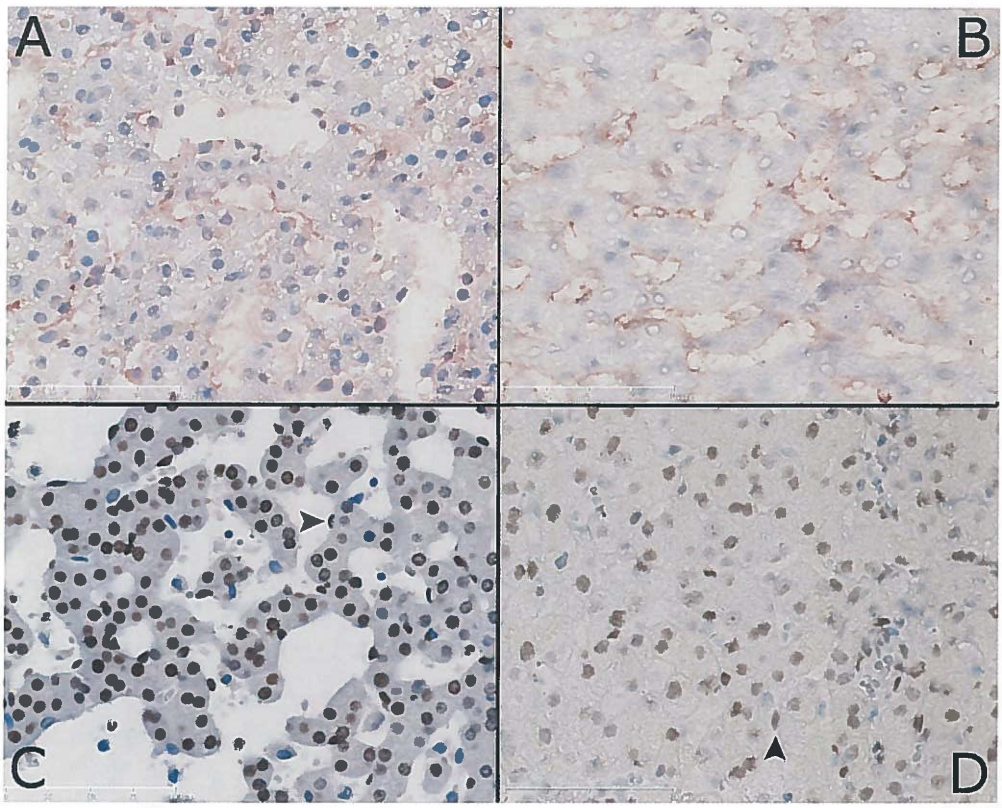

Figure.6. Localization of leptin receptor (LR), adiponectin receptor 2 (AdiR2) and phospho-STAT3 (p-STAT3) in the inflammatory hepatocelluar adenoma

A: LR (red) and **B:** AdiR2 (red) in tumor endothelial cells in a sinusoidal pattern, 400 ×;

C: p-STAT3 (brown) in tumor located on the nuclei of hepatocyes and sinusoidal endothelial cells (arrow head), 400 ×;

D: p-STAT3 (brown) in non-tumor liver located on nuclei of hepatocytes and few sinusoidal endothelial cells (arrow head), 400 ×.

LR gene expression in LMD tumor and LR protein expression in tissue

As depicted in Figure 7 B, CD34 gene expression in LMD-tumor hepatocyte samples is decreased to 50% compared to the CD34 gene expression in whole tumor samples. LR gene expression in LMD-tumor hepatocyte samples decreased by only 10% compared to the whole tumor samples suggesting that LR is expressed by hepatocytes. Results of the western blot analysis of LR (Figure 8) showed that LRb, the long form which can activate JAK2/STAT3 pathway, was

overexpressed in the tumor, more than the corresponding distant liver samples in cases No.1 and No.3. In both cases expression of the short form of LR expression was more pronounced in the distant liver than in the tumor. There is no obvious difference in the expression of the LRb isoform in case No.2 (Figure 8).

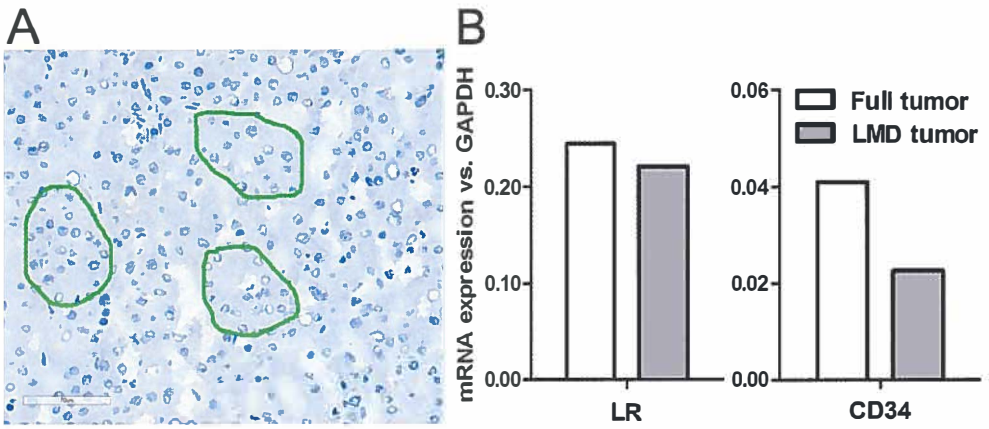

Figure 7. LR gene expression in the LMD-tumor hepatocytes and the full tumor.

A: Clusters of hepatocytes (green circles) in the tumor of one IHCA case were selected for LMD, followed by RNA isolation. Hematoxylin staining, 400×;

B: LR and CD34 relative gene expression in the LMD-tumor hepatocytes and the full tumor tissues. mRNA levels shown are relative to GAPDH and detected by quantitative RT-PCR as described in MATERIALS AND METHODS.

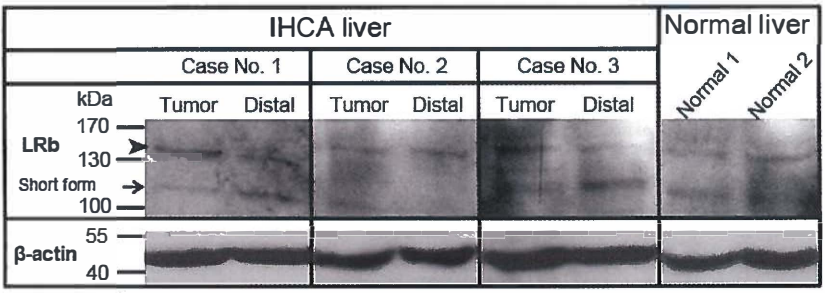

Figure 8. LRb protein expression in IHCA and normal liver samples. Western blot analysis of LRb in 3 pairs of IHCA cases including tumor and corresponding distant liver samples and 2 samples of normal liver.

Arrow head: size (in kDa) of LRb protein. Arrow: short form of LR.

Leptin stimulation of Endothelial Cells:

Increased gene expression of LR and IL-6 after leptin stimulation

Following leptin stimulation for 24 hours, HUVEC showed an increase in both LR and IL-6 gene expression compared to control (vehicle), (p= 0.033 and 0.044 respectively). hHSEC underwent leptin stimulation for 12 hours, whereafter a significant increase of IL-6 gene expression was observed (p=0.021) but no increase of LR (Figure 9).

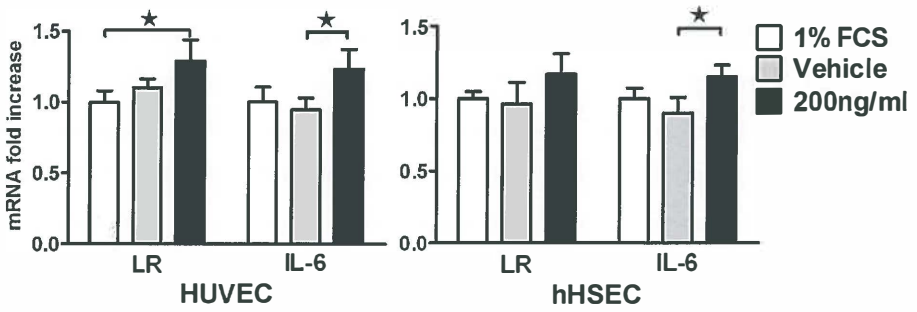

Figure 9. Leptin receptor (LR) and interleukin-6 (IL-6) mRNA expression in endothelial cells upon incubation with leptin. LR and IL-6 mRNA shown as fold increased expression in human umbilical vein endothelial cells (HUVEC) and human hepatic sinusoidal endothelial cells (hHSEC) following leptin stimulation.

HUVEC and hHSEC were exposed to leptin for 24 h and 12 h respectively and mRNA levels are shown relative to 1% fetal calf serum (FCS) and vehicle control, detected by qRT-PCR as described in MATERIALS AND METHODS. Values are mean ± SD of three analyses. ★ = p<0.05.

DISCUSSION

It is well established that IHCA is associated with obesity (6, 9, 10). Thus far, the pathogenetic mechanisms behind this association have been rather elusive. To explore the possible influence of obesity on IHCA we here investigated the expression profiles of leptin and adiponectin and their respective receptors as these represent the 2 most important adipokines systems associated with obesity. Moreover, leptin has also been established as a proinflammatory and growth

stimulating cytokine (16, 18). In addition to this, we analysed the status of the signaling molecules downstream of leptin-LR activation.

Leptin and adiponectin gene expression was not detectable in tumor and non-lesional liver by 2 different methods whereas ample expression of both factors was observed in adipose tissue that was included as controls. This observation confirms that adiponectin and leptin are primarily produced in adipose tissue and that hepatic synthesis of these growth factors is immeasurably low if existent at all (16). Failure to detect adiponectin mRNA in human liver has been described before (19) and evidence for hepatic leptin synthesis has sofar only been described in HCC cell lines (Huh7 and HepG2) and in rodents and avian species (22-24).

By immunohistology we observed leptin and adiponectin expression in Kupffer cells but not in other liver cell constituents. Taken these data together, they imply that this liver associated leptin and adiponectin is derived from an exogenous source, e.g., by uptake from serum.

With regard to the receptors, we found a higher gene expression level of LR and lower level of AdiR2 in IHCA compared to the adjacent non-tumorous liver in which both receptors were mainly expressed by tumor EC, both in the tumor sinusoids and tumor vessels. In the additional LMD studies of tumor hepatocytes gene expression indicated hepatocytic expression of LR. In the *in vitro* leptin stimulation studies using endothelial cell cultures we found increased LR gene expression in HUVEC but not in hHSEC. This *in vitro* finding may parallel EC characteristics in IHCA because despite the sinusoidal pattern in IHCA, the tumor EC of IHCA express CD34. This condition is designated as sinusoidal capillarization, rendering VEC characteristics to these tumor EC (25-27).

The *in vitro* finding that leptin can stimulate endothelial LR expression, however minor, may explain the increased LR expression in IHCA. Overweight individuals have an aberrant serum profile of adipokines consisting of hyperleptinemia and hypoadiponectinemia (16, 28). In our IHCA patients who are overweight and obese, hyperleptinemia may stimulate LR expression in the microvasculature that is embedded in the tumor. The absence of significant LR increase in the non-lesional liver may be due to the microenvironmental conditions

that do not render the CD34 negative SEC prone to changes in the non-lesional liver (29).

A particular finding in this study is that the increased level of tumor LR is mainly generated by patients with a BMI < 30 which is suggestive of a leptin-resistance condition, a well-known phenomenon in obese patients. This phenomenon mainly concerns the central nervous system's resistance to leptin and results from several mechanisms including impaired active transport of leptin across the blood-brain barrier, decreased LR expression and defective leptin signaling due to the absence of negative feedback by SOCS3 (30, 31). The possibility of *hepatic* leptin resistance has been suggested based on various observations in animal models, involving increased SOCS3 and downregulation of LR in dietary-induced obesity (16, 32). We did not find significant differences in the gene expression level of SOCS3 between tumor and non-tumor tissue nor compared with normal liver. This finding suggests the presence of a hepatic leptin-resistance like condition in IHCA patients with BMI ≥30 who showed a lower potential of LR induction. This hepatic leptin-resistance like condition may become manifest with increasing severity of obesity which corroborates the association of obesity with IHCA.

The presence of nuclear p-STAT3 expression confirms the activation of the leptin/LRb pathway. Of note, there is a more prominent LRb protein expression in the tumor than in the distant liver based on the western blot analysis. Leptin binding to LRb activates JAK2 phosphorylation resulting in a cascade of several downstream signaling effects, among others STAT3 activation (33). However, the latter is also the result of the mutational changes found in IHCA while these mutations were not paralleled by an increased gene expression of IL-6 in the tumor itself (7). Increased serum IL-6 is however frequently found in obese patients along with increased TNF-α (17). Our results also showed that following leptin stimulation both HUVEC and hHSEC showed increased IL-6 expression. Obesity related increased serum IL-6 may have an additional effect on the mutated IL-6 receptor while hyperleptinemia in these patients could induce STAT3 activation through the increased LR expression in the tumor. Moreover, we also found nuclear p-STAT3 expression in the non-lesional tissue of the IHCA patients, signifying STAT3

activation in the part of the liver without IHCA-related mutational changes, possibly representing obesity related STAT3 activation.

In IHCA nuclear p-STAT3 expression was found in tumor EC and hepatocytes. On immunohistology LR was mainly present in EC while the additional LMD studies of tumor hepatocytes indicated hepatocytic expression of LR. The nuclear p-STAT3 expression on tumor EC and hepatocytes confirms the activation of STAT3 in these cells that, based on our findings, may partly be due to leptin/LR binding. This binding may influence tumor growth directly via the JAK2/STAT3 pathway. Moreover, based on the firmly established indispensable role of SEC on hepatocyte proliferation, activation of tumor EC following leptin/LR stimulation will in all likelihood contribute to the growth of IHCA (34, 35).

Several studies have documented the potential of the leptin/LR pathway in inducing tumor growth, via stimulation of angiogenesis but also proliferation of tumor cells (16,18,36). Hence, one of the most important possible consequences of increased LR expression in IHCA is probably the facilitation of tumor growth which may also be a contributing factor in the transformation of HCA into HCC.

In summary, our findings of a higher gene expression level of LR in IHCA and specifically in tumor associated endothelial cells, and a lower expression of AdiR2 compared to its non-lesional hepatic counterpart, combined with the potential of leptin-stimulated EC to increase IL-6 and LR expression have provide a first pathogenetic link between obesity and IHCA. In the light of the increasing recognition of the roles of leptin and adiponectin in carcinogenesis in obese individuals these findings may stimulate further studies in the malignant transformation of HCA into HCC in the context of obesity.

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CHAPTER 4

Effects of leptin on endothelial cell proinflammatory activation status and leukocyte interaction

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Manuscript in preparation

ABSTRACT

Inflammatory type hepatocellular adenoma (IHCA), one of the 4 subtypes of hepatocellular adenomas (HCA) is associated with high body mass index (BMI) and is histologically characterized by the presence of inflammatory infiltrates, dilated sinusoids and ductular reaction. Leptin, one of the main adipokines secreted by adipose tissues, is positively related with a high BMI, and leptin has been regarded as a proinflammatory agent. Whether a relation exists between proinflammatory actions of leptin and leukocyte infiltration in IHCA is unknown.

We investigated the influence of leptin on leptin receptor (LR) induced signal transduction in human umbilical vein endothelial cells (HUVEC) and human hepatic sinusoidal endothelial cells (hHSEC). We showed that both types of cells expressed LR, and that signal transducer and activator of transcription 3 (STAT3) was rapidly activated upon leptin stimulation. In accordance, mRNA of suppressor of cytokine signalling 3 (SOCS3) was augmented upon short term exposure to leptin. Both in HUVEC and hHSEC mRNA levels of intercellular adhesion molecule-1 (ICAM-1) were upregulated, though to a minor extent. Furthermore, in HUVEC endothelin-1 and nitric oxide synthetase 3 mRNA levels were slightly increased. Leptin incubation also increased cellular surface expression of ICAM-1 protein in HUVEC, but did not lead to an increase in HL60 adherence to HUVEC in a leukocyte-endothelial adhesion assay. No effect of leptin on leukocyte adhesion could be observed in conjunction with tumor necrosis factor α (TNFα) induced activation.

In conclusion, leptin/LR is activated properly in endothelial cells. Leptin exposure resulted in upregulation of mRNA and protein levels of ICAM-1, though to a minor extent, and did not affect leukocyte-endothelial adhesion. In IHCA, special local context or other cytokines may be involved in the molecular basis of inflammatory process and interacted with leptin lead to inflammatory infiltration in IHCA.

INTRODUCTION

Leptin, a 16kDa cytokine/hormone, is one of the major adipokines synthesized by adipose tissues, regulating food intake and energy expenditure in the brain and acting as proliferation, angiogenesis and tumorigenesis factor in peripheral tissues (1, 2). The proinflammatory effects of circulating leptin have been demonstrated in many studies. For example, Mabuchi *et al* found that increased white blood cell counts are present in obese people with elevated serum leptin (3). Another study by De Rosa et al showed that leptin upregulated the production of C reactive protein (CRP) by human coronary artery endothelial cells (HAEC), which may increase the risk of cardiovascular disease (4). To study the proinflammatory role of leptin *in vivo*, leptin (*ob/ob*) and leptin receptor *b* (*db/db*) deficient mice have been applied. Siegmund et al found that in chronic colitis production of proinflammatory cytokines and inflammatory cell infiltration were suppressed in colonic tissue of *ob/ob* mice compared with wild type mice (5). Furthermore, Tanaka et al showed that deficiency of leptin signaling reduced unilateral ureteral obstruction induced renal macrophage infiltration via suppression of expression of monocyte chemoattractant protein-1 in kidney of *ob/ob* and *db/db* mice (6). The mechanisms of proinflammatory cell activation by leptin are still not well understood.

Inflammatory type hepatocellular adenoma (IHCA), a main subtype of hepatocellular adenoma (HCA), is observed more frequently in female patients with high body mass index (BMI≥25) as demonstrated by several studies (7, 8). Overweight individuals have more ectopic fat with dysfunctional adipose tissues, especially visceral adipose tissues, that release adipokines including leptin, which can target the liver directly via the portal vein and serum leptin is positively related to increased BMI (9, 10). Leptin receptor (LR) is expressed on vascular endothelial cells, sinusoidal endothelial cells (SEC) and some cells of the immune system besides on blood-brain barrier composed of central microvessels and neurons of the hypothalamus (1, 11, 12).

Since hyperleptinemia is presumably part of the microenvironment of IHCA due to the overweight condition of these patients, our previous study investigated LR expression and localization in the tumor and local environment. We found that

mRNA levels were upregulated in IHCA and LR was expressed by SEC (manuscript in preparation). We assumed that leptin may play a proinflammatory role via binding to LR on SEC in IHCA, contributing to the presence of inflammatory cells which is a characteristic feature of IHCA (13).

The purpose of this current study was to test our hypothesis that serum leptin can stimulate inflammation via LR activation in endothelial cells. Hence, we mimicked leptin stimulation by exposing human umbilical vein endothelial cells (HUVEC) and human hepatic sinusoidal endothelial cells (hHSEC) to leptin to get insight into the influence of leptin on endothelial cells. We studied activation of leptin/LR signaling, genes and proteins regulated by leptin. We further performed leukocyte-endothelial adhesion assays. Lastly, we investigated whether leptin acted in conjunction with tumor necrosis factor α (TNFα), which is also increased in overweight patients (14).

MATERIALS AND METHODS

Cell and culture conditions

HUVEC and hHSEC were purchased from Lonza Walkersville Inc (Walkersville, USA) and ScienCell Research Laboratories (San Diego, USA) respectively and cultured in the UMCG Endothelial Cell Faculty. HUVEC were used at the fifth or sixth passage and hHSEC were used at the fourth passage. HUVEC were cultured in EGM2-MV (CC 3156) with 5% fetal calf serum (FCS), penicillin, and endothelial cell growth factor. All these reagents were bought from Lonza Walkersville Inc. hHSEC were maintained in ECM (No. 1001) with 5% FCS, penicillin/streptomycin, and endothelial cell growth supplement, all reagents were obtained from ScienCell Research Laboratories. HL60 (kindly provided by Prof. Georg H. Fey, University of Erlangen, Germany) was grown in RPMI 1640 medium with 10% FCS.

Cell incubation conditions

Human recombinant leptin was purchased from R&D Systems (Abingdon, UK) and human tumor necrosis factor α (TNFα - Beromun) from Boehringer Ingelheim (Germany).

HUVEC and hHSEC were seeded at 70% confluence in 24-well plates for RNA isolation, 12-well plates for flow cytometry analysis or leukocyte adhesion assay and 6-well plates for western blot. All plates were coated with gelatine or fibronectin for HUVEC and hHSEC culturing before seeding respectively. After culturing for 12 h, the cells were starved with 1%FCS for 16 h. Then the cells were exposed to 200 ng/ml respectively 50 ng/ml leptin for 15 min, 60 min, 12 hours and 24 hours. Endothelial cells with 1%FCS and 2% 20mM Tris-HCL exposure were set as control and vehicle control wells respectively. HUVEC activated with TNFα (10 ng/ml) were used as positive control for cell adhesion molecule induction as analyzed by flow cytometry. A concentration range of TNFα (0.01 - 10 ng/ml) and 200 ng/ml leptin were added to HUVEC for leukocyte-endothelial adhesion analysis. Each condition was executed in triplicate.

Quantitative RT-PCR

Total RNA of cells was isolated with the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands), according to the protocols provided with the kit. After checking RNA integrity by electrophoresis and analyzing RNA concentration by Nanodrop ND-100 spectrophotometer (NanoDrop Technologies), 11.5 µl of total RNA of each sample was reverse transcribed into cDNA using superscript III RT (Invitrogen, Bleiswijk, the Netherlands). 2 ng cDNA was applied for each PCR reaction. Primers for real-time PCR were purchased from Applied Biosystems (Nieuwekerk a/d IJssel, the Netherlands) (Table 1). PCR reactions were performed in an ABI 7900HT Sequence Detector (Applied Biosystems, CA, USA), using the following conditions: 15 min at 95°C, 40 cycles of 15s at 95°C and 60s at 60°C. Duplicate real-time PCR analyses were performed for each sample, yielding an average Ct value per sample. Gene Ct values were compared to hGAPDH as housekeeping gene, and yielded the relative gene expression values. Each sample was compared to

1%FCS condition and averaged values of triplicates of one condition as mean fold increase ± standard deviation (SD).

Table 1 Primers used for quantitative RT-PCR

Gene	assay ID
hGAPDH	Hs99999905_m1
hHGF	Hs500300159_m1
hPGF	Hs00182176_m1
hVEGFA	Hs00173626_m1
hVEGFR1	Hs01052936_m1
hVEGFR2	Hs00176676_m1
hICAM-1	Hs00164932_m1
hVCAM-1	Hs00365486_m1
hEDN1	Hs00174961_m1
hNOS3	Hs00167166_m1
hANGPT-1	Hs00181613_m1
hANGPT-2	Hs00169867_m1

Flow cytometry for intercellular adhesion molecule-1 (ICAM-1) protein expression

HUVEC were harvested at 12 h and 24 h of exposure to 200 ng/ml leptin respectively 10 ng/ml TNFα, then the cells were stained for ICAM-1 protein expression using monoclonal mouse anti-human ICAM-1 (Abcam, ITK Diagnostics, Uithoorn, the Netherlands) at 4°C for 1 hour. After washing with PBS, the cells were incubated with FITC-labeled rabbit-anti-mouse secondary antibody (DAKO, Glostrup, Denmark), diluted 1:40 in 5% rabbit serum/PBS at 4°C for 30 minutes. After the final washing, cells were resuspended in PBS and analyzed using a BD FACS Calibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). The living cells were gated to determine the Mean Fluorescence Intensity (MFI) for protein levels of ICAM-1.

Quantification of leukocyte-endothelial cell interaction

HUVEC at 100% confluence were used for leukocyte-endothelial interaction after exposure to 200 ng/ml and 50 ng/ml leptin, or co-incubation with leptin and TNFα for 12 h respectively 24 h. Cells stimulated with 10 ng/ml TNFα were used as positive control and medium incubated cells as negative, quiescent control.

HL60 were labeled with 10 µg/ml Hoechst (Invitrogen, Bleiswijk, the Netherlands) for 10 min, and resuspended in HL60 medium at a concentration of 500,000 cells/ml after washing 3 times. HUVEC were co-incubated with 1 ml HL60 suspension for 1 hour followed by removing the medium. By rigorously washing the wells 3 times with PBS, non-adherent HL60 were washed away and 80 µl trypsin was added to each well for 3-4 min to loosen up all cells. Trypsinized samples were collected in FACS tubes with 50 µl FCS and the wells were washed with 100 µl PBS another 3 times to collect all cells (15).

Cells were quantified using a MACSQuant machine (Miltenyi Biotec, Leiden, the Netherlands). For live cell populations of each sample, forward scatter vs. side scatter (FSC/SSC; both in log scale) and VioBlue-A vs. FITC-A (both in log scale) dot plots were acquired for 50 µl volume per sample. Data analysis was done with Kaluza software by setting the gate for adherent HL60 on VioBlue-A vs. FITC-A dot plot.

Western blot

The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate and 0.1% SDS) containing protease inhibitor cocktail (Roche Diagnostics, Almere, the Netherlands). After measuring the protein concentration by DC protein Assay following the manual (BIO-RAD, Veenendaal, the Netherlands), 20 µg protein of each sample was separated on 10% SDS-PAGE or 7% SDS-PAGE for LR protein detection and transferred to nitrocellulose membranes (GE Healthcare Europe GmbH, Diegem, Belgium). Membranes were blocked with 5% non-fat dry milk (Campina, Woerden, the Netherlands) or 5% BSA (Sigma-Aldrich, St Louis, USA) for phospho-STAT3 (p-STAT3) antibody in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) for 1.5 hours, and then probed with the following primary

antibodies: rabbit anti-LR polyclonal antibody (Santa Cruz, Bioconnect, Huissen, the Netherlands) diluted 1:400 in 5% non-fat dry milk/TBST, rabbit anti-p-STAT3 polyclonal antibody (Cell Signaling, Bioke, Leiden, the Netherlands) diluted 1:800 in 5% BSA/TBST, monoclonal mouse anti-β-actin (Abcam, ITK Diagnostics, Uithoorn, the Netherlands) diluted 1:4,000 in 5% non-fat dry milk/TBST. The horseradish peroxidase-conjugated secondary rabbit anti-mouse and goat anti-rabbit antibodies (Dako, Glostrup, Denmark) used for detection were diluted 1:2,000 in 5% non-fat dry milk/TBST respectively 5% BSA/TBST (p-STAT3) and visualized using the Pierce ECL2 chemoluminoscence detection kit (Thermo Scientific, Rockford, USA). After detection of p-STAT3, the membrane was washed in TBST and submerged in stripping buffer (25mM glycine, 1%-2% SDS, pH2.0, LI-COR Biotechnology, Leusden, the Netherlands) for 20 min at room temperature. The membrane was blocked with 5% non-fat dry milk/TBST for 1.5 h, and then probed with rabbit anti-STAT3 polyclonal antibody (Millipore, Merck, Schiphol-Rijk, the Netherlands) diluted 1:1,000 in 5% non-fat dry milk/TBST, followed by second antibody and detection with ECL2 as described above. Quantification of the relative protein expression levels of LR, STAT3, and p-STAT3 was performed by comparison of the signals with β-actin signal using Image J software. These total procedure quantification analyses of triplicate samples were averaged as mean ± SD.

Immunofluorescence

Coverslips 12 mm in diameter were bought from Menzel-Gläser (VWR International, Dublin, Ireland). The cell suspension was seeded on coverslips one day before start of a 16 h period of cell starvation. Cells on the coverslips were stimulated with leptin at 200 ng/ml for 15 min and 60 min, and TNFα at 10 ng/ml for 60 min as positive control. After fixation with methanol/acetone (1:1) for 10 min on ice, samples were blocked in 3% BSA in PBS for 30 min at room temperature, then incubated with p-STAT3 (1:100) in 0.5% BSA/PBS for 1.5 h, followed by secondary goat anti-rabbit-Alexa 488 antibody (1:400) (Life Technologies Europe BV, Bleiswijk, the Netherlands) for 1 h, mounted with aquapolymount with DAPI

(Ploysciences. Inc, Warrington, PA) on a microscope slide, and analyzed by fluorescence microscopy (Leica, Germany).

Statistics

Data were analyzed with SPSS statistics 18 software (SPSS, Chicago, IL, USA) and presented as mean ± SD. One-way ANOVA with Bonferroni correction was performed when comparing means among more than two groups. Significance was set as $p < 0.05$.

RESULTS

HUVEC express leptin receptor

To determine whether LR was expressed and the receptor signalling pathway was activated in HUVEC, we first analyzed the protein expression by western blot. LR was indeed expressed in quiescent HUVEC. Both the short form and the long form of LR were expressed by HUVEC. Both are recognized by the antibody used (Figure 1 A). The long form (*b* form) of LR is crucial for leptin action via STAT3 and other signalling pathways (9). No increase in LR (long form, 125 kDa) protein was seen in HUVEC after leptin activation for 24 h compared with 1%FCS control (Figure 1 A). Activation of STAT3, represented by a p-STAT3 signal in western blot, could not be detected in leptin stimulated HUVEC, yet was detectable in TNFα stimulated HUVEC used as control (Figure 1 B).

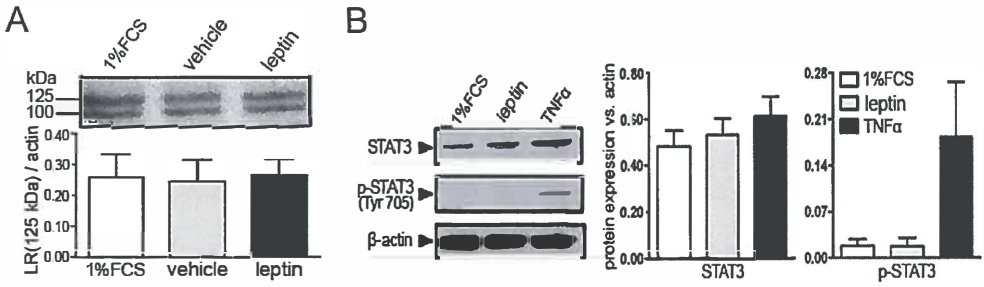

Figure 1 Leptin receptor (LR), STAT3 and phospho-STAT3 (p-STAT3) protein expression upon incubation of HUVEC with leptin for 24 h.

A: Western blot of LR and relative protein levels of LR expressed by HUVEC after 24 h incubation with leptin at 200 ng/ml.

B: Western blot of STAT3 and p-STAT3 and relative protein levels of STAT3 and p-STAT3 expressed by HUVEC after 24 h incubation with leptin at 200 mg/ml and TNFα 10 ng/ml respectively.

Values are mean ± SD of three analyses. Arrow head, size of protein of interest

Leptin activates p-STAT3 in 15 min incubation

Since we failed to observe STAT3 phosphorylation induced by leptin upon 24 h incubation, we exposed HUVEC to leptin for a short time. Immunofluorescence detection of p-STAT3 now showed a major increase in p-STAT3 accumulation in the nuclei of HUVEC activated by leptin for 15 min and 60 min. Compared with 15 min, less nuclei showing p-STAT3 accumulation were present in HUVEC exposed to leptin for 60 min or to TNFα (Figure 2 A). These data were corroborated by western blot for p-STAT3, which showed an increase of p-STAT3 expression at 15 min and 30 min incubation with leptin (Figure 2 B). This signal diminished at 60 min incubation, which might be related to suppressor of cytokine signalling 3 (SOCS3) upregulation. SOCS3 is an immediate early response gene of leptin-LR signalling, which inhibits leptin-LR signal pathway (9, 16) and whose mRNA was upregulated 8 fold at 15 min incubation and 10 fold at 60 min incubation with leptin (Figure 2 C).

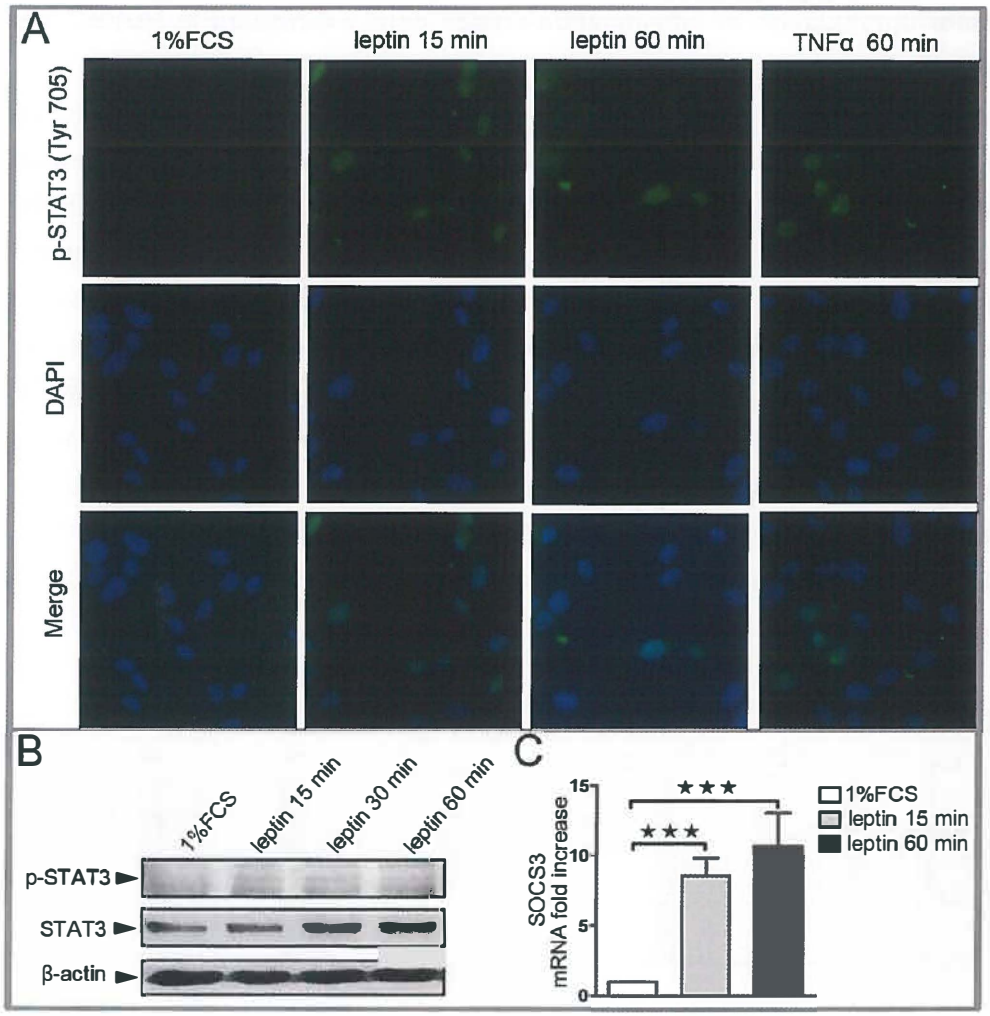

Figure 2 Phospho-STAT3 (p-STAT3) is activated in HUVEC within 15 min after start of incubation with leptin.

A: Immunofluorescence of p-STAT3 (green nuclei) expressed by HUVEC incubated with 1%FCS, leptin at 200 ng/ml, and TNFα at 10 ng/ml;

B: Western blot of p-STAT3 and STAT3 protein expressed by HUVEC incubated with 1%FCS, leptin at 200 ng/ml. Arrow head, size of protein of interest;

C: SOCS3 mRNA levels in HUVEC upon incubation with leptin at 200 ng/ml for 15 min and 60 min. ★★★p<0.001.

Minor upregulation of inflammation-related gene expression in HUVEC and hHSEC by exposure to leptin

After showing that leptin-LR is properly activated in our experimental set up, we aimed to measure the expression of genes related to inflammation, proliferation and angiogenesis (Table 1), in HUVEC as well as in hHSEC based on the assumption that leptin-LR was also functional in hHSEC. ICAM-1, Endothelin-1 (EDN1) and nitric oxide synthetase 3 (NOS3) were upregulated with statistical significance in HUVEC after stimulation with 200 ng/ml leptin for 24 h compared with vehicle control (p<0.05) (Figure 3 A). None of the other genes related to proliferation and angiogenesis (Table 1) showed a difference in expression levels after leptin stimulation (data not shown). Similarly, only ICAM-1 was upregulated after leptin stimulation of hHSEC for 12 h compared to vehicle control (p<0.05) (Figure 4). No change of gene expression could be observed in hHSEC with exposure to leptin for 24 h (data not shown).

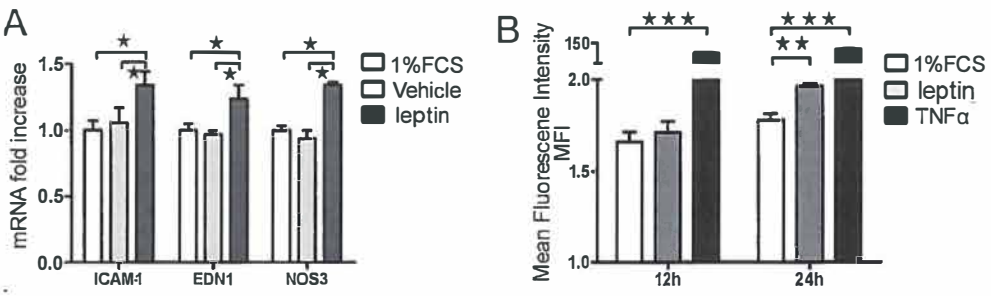

Figure 3 Inflammation related gene and protein expression by HUVEC in response to leptin exposure.

A: Inflammation related gene expression in HUVEC after 24 h incubation with leptin at 200 ng/ml. mRNA levels were analysed by quantitative RT-PCR, mRNA fold increase shown is compared with 1%FCS as control, White column, 1%FCS; gray column, vehicle control; black column, 200 ng/ml leptin.

B: ICAM-1 mean fluorescence intensity in HUVEC upon incubation with leptin at 200 ng/ml for 12 h and 24 h as assessed by flow cytometric analysis. White column, 1%FCS; gray column, 200 ng/ml leptin; black column, 10 ng/ml TNFα stimulation.

Values are mean ± SD of three analyses. ★p<0.05; ★★ p<0.01; ★★★ p<0.001.

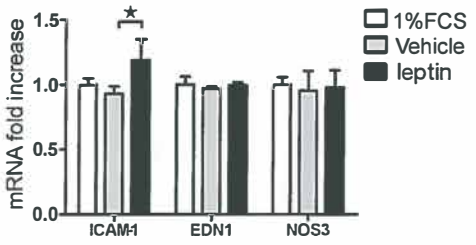

Figure 4 Inflammation related gene expression by hHSEC incubated with leptin for 12h. mRNA levels were analysed by quantitative RT-PCR, mRNA fold increase shown is compared with 1%FCS as control, Values are mean ± SD of three analyses. White column, 1%FCS; gray column, vehicle control; black column, 200 ng/ml leptin. ★p<0.05.

Leptin induces surface expression of ICAM-1

To further investigate whether the ICAM-1 gene expression induction was followed by an increase in protein expression, we performed flow cytometric analysis of ICAM-1. TNFα stimulated HUVEC, serving as control condition, significantly upregulated ICAM-1 expression from MFI 1.8 ± 0.2 to 99.7 ± 2.2. HUVEC exposed to 200 ng/ml leptin for 24 h increased ICAM-1 expression, from MFI 1.8 ± 0.2 into 2.0 ± 0.01. This effect was statistically significant, yet the increase is considered minor. No change was observed in HUVEC incubated with leptin for 12 h (Figure 3 B).

Leptin stimulation did not affect HL60 adherence to endothelial cells

To explore possible consequences of endothelial activation induced by leptin which we did not uncover by gene expression analysis, we studied leukocyte-endothelial adhesion as a biological functionality. As expected, the number of HL60 cells adherent to the HUVEC monolayer after TNFα incubation was significantly increased compared to control. No change in number of HL60 cells adherent to the HUVEC after leptin incubation could be observed, either at 12 h or at 24 h leptin activation of HUVEC (Figure 5 A).

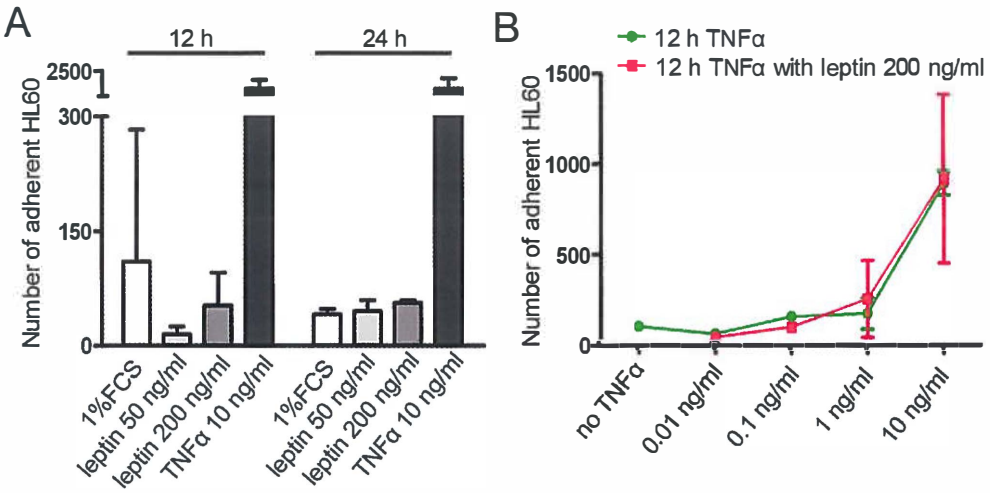

Figure 5 Leukocyte-endothelial adhesion in response to leptin, TNFα and leptin/TNFα respectively.

A: Number of HL60 cells adherent to HUVEC after pre-incubation with leptin were quantified as described in 'Materials and Methods'. White column, 1%FCS; light gray column, 50 ng/ml leptin; dark gray column, 200 ng/ml leptin; black column, 10 ng/ml TNFα stimulation;

B: Adherent HL60 cell numbers after HUVEC co-incubation with TNFα and leptin for 12 h. Circles, TNFα; squares, TNFα plus 200 ng/ml leptin. Values are mean ± SD of three analyses.

Leptin does not enhance the effects of TNFα in adhesion

Besides hyperleptinemia, increased serum levels of TNFα exists in overweight patients (14), hence we co-incubated leptin with different concentrations of TNFα to investigate the influence of leptin on endothelial cell adhesion properties induced by TNFα. As shown in Figure 5 B, obvious increased leukocyte adhesion could be observed in HUVEC activated by TNFα compared to control, in a concentration dependent manner. Leptin co-incubation did not change the adhesion effects induced by TNFα (Figure 5 B).

DISCUSSION

Since leptin is a proinflammatory adipokine in peripheral tissue and hyperleptinemia is present in overweight patients who represent a major group of

IHCA patients, we aimed to explore the possible mechanisms of inflammatory cell infiltration in IHCA induced by leptin. Based on our previous finding that mRNA of LR was upregulated in IHCA and LR was expressed by SEC (manuscript in preparation), we investigated the activation of leptin-LR in endothelial cells, gene and protein expression regulated by leptin, and *in vitro* leptin effects on leukocyte adhesion to endothelial activation. We found that leptin activated LR properly in EC, and that leptin could upregulate mRNA and protein levels of ICAM-1, though to a minor extent. Furthermore, leptin activation of EC did not lead to a significant change in leukocyte adhesion.

LR has six isoforms (*a* to *f*), and is classified into a secreted, short and long form receptor, in which the long form (*b* form) receptor is crucial for leptin signalling mainly via activation of the Janus kinase 2 (JAK2)/STAT3 pathway (1, 9). In HUVEC and hHSEC (data not shown), both the short and long form of leptin receptor proteins are expressed without any (leptin) stimulation.

Phospho-STAT3, representing activation of STAT3, was absent in HUVEC upon 24 h incubation by leptin, but was present in HUVEC activated by TNFα. However, shorter incubation with leptin for 15 min, a major increase in p-STAT3 accumulation in the nuclei of HUVEC as was made visible by immunofluorescence. Then the signal for p-STAT3 accumulation declined at 60 min incubation with leptin, which was corroborated by western blot showing p-STAT3 expression. These data indicate that leptin-LR is activated properly in HUVEC in a short period, which corroborates a previous study mentioning that the p-STAT3 is activated after 15 min exposure to leptin followed by a decline caused by feedback inhibitors of this pathway that were not further specified (17). One well-known inhibitor of the leptin-LR signalling pathway is SOCS3, which is one of STAT3-regulated genes (9, 18). In our study, we found that SOCS3 is indeed an immediate early response gene of leptin-LR signalling, whose mRNA level is remarkably upregulated after 15 min leptin incubation compared to control. This quick response of SOCS3 induced by leptin might lead to inhibition of STAT3 activation, and may thus be responsible for lack of p-STAT3 at later time points of leptin incubation in EC.

With the proper activation of leptin-LR in HUVEC, we detected inflammation related gene expression induced by leptin. ICAM-1, EDN1 and NOS3 were

upregulated at 24 h incubation compared to control in HUVEC, not yet at 4 h and 12 h incubation with leptin (data not shown). In addition, interleukin-6 (IL-6) was upregulated as we showed in our previous study (manuscript in preparation), though this upregulation was also minor (1.5 fold). Moreover, we found that higher MFI for ICAM-1 expression in HUVEC upon leptin exposure for 24 h compared to control, yet the upregulation was minor compared to TNFα induced upregulation. Although the induced changes were quite minor, the outcome is consistent with some recently published studies (19, 20). For instance, Manuel-Apolinar et al exposed isolated endothelium from the thoracic aorta of rats to leptin and found an increased mRNA expression of LR (long form) and ICAM-1 around 2 fold compared to control at 24 h incubation with 10 nM leptin (160 ng/ml) (20). A maximum of 5 fold increase of ICAM-1 was observed at 48 h exposure to the same concentration, which is a time frame we did not study. Our findings indicate that minor ICAM-1 protein upregulation is in accordance with minor mRNA upregulation of ICAM-1 observed at 24 h incubation with leptin.

Since we only studied a few genes, there was a possibility that we missed many that may be involved in inflammatory activation, thus we decided to study the functional consequences of EC exposure to leptin by executing leukocyte-endothelial adhesion assays. In this assay, we also did not observe changes in the number of HL60 cells adhering to HUVEC under the influence of leptin. Also, no effect of leptin on leukocyte adhesion induced by TNFα was observed.

Kralisch S et al applied monocyte adhesion to HUVEC cultured in primary differentiated human adipocytes conditioned medium containing 0.25 ng/ml TNFα and 10.30 ng/ml leptin. Adipocytes conditioned medium induced monocyte adhesion could be partly inhibited by TNFα-neutralizing antibody pre-incubation with HUVEC, while even at 1000 ng/ml leptin incubation could not induce monocyte adhesion (21). These findings may suggest that a minute concentration of TNFα might play an essential role in adhesion. In obese patients, Moschen et al showed the serum TNFα declines from 2.36 ± 0.08 pg/ml to 0.8 ± 0.03 pg/ml at 12 months after weight loss (14). The results of these 2 studies denote that serum TNFα is increased and exert proinflammatory roles in obese individuals. Other studies mentioned that leptin could stimulate TNFα production by circulating monocytes in

man (22, 23), which suggest that leptin might induce leukocyte-endothelial cell adhesion via the production of proinflammatory cytokines by leukocytes, a process we did not investigate.

The hHSEC we used as *in vitro* model represent normal hepatic SEC, whose *in vivo* context is different from HUVEC. The patterns of responses to leptin in hHSEC and HUVEC could potentially be different. However, in this study, the influences of leptin on hHSEC (data not shown) and HUVEC are almost the same. A previous study by Liu et al supports our outcomes. They found that arterial and venous ECs change their different *in situ* patterns of response to TNFα quickly into a common EC culture phenotype when both ECs are detached from the umbilical cord vessel walls and put them into cell culture for 72 hours (24).

In overweight patients with non alcoholic steatohepatitis or obese individuals, several reports have revealed that many inflammatory activators are present in their serum, including lipopolysaccharide (LPS), CRP and adiponectin, besides leptin and TNFα (1, 25). These cytokines may all interact with leptin. Vaughan et al showed that 10 ng/ml leptin can prominently enhance the impact of LPS on stimulating the expression of IL-6 in human monocytes (26). Moreover, less SOCS3 was bound to functional leptin receptor, and less protein tyrosine phosphatase-1 B, another inhibitor of leptin signalling pathway (18, 27), was generated in livers of adiponectin deficient mice compared to wild type mice (28). Similarly, obese people usually have hypoadiponectinemia besides hyperleptinemia (1), hence the indirect inhibition factors (e.g. adiponectin) for leptin/LR signalling failed to disturb the aggressive effects of leptin. However, in individuals, the microenvironment is likely much more complicated than what is present in this *in vitro* study, there may exist other triggers which can increase the proinflammatory role of leptin in IHCA. For instance, dilated sinusoid with some sinusoidal capillarization might change the microcirculation in IHCA (13, 29-31), which might favour leukocyte adhesion in IHCA. As Liu et al demonstrated that response differences between arterial and venous ECs are dependent on their different *in situ* contexts, we likely could not mimic the real microenvironment of IHCA in our *in vitro* set up (24).

In summary, leptin properly activated leptin/LR signaling in endothelial cells, and leptin upregulated mRNA and protein level of ICAM-1, but the effects were minor and leptin could not induce leukocyte-endothelial adhesion. In IHCA, more leads regarding the molecular basis of the IHCA inflammatory process, such as hypoadiponectinemia, special local context, or other cytokines, interact with leptin and should be sought in inflammatory infiltration in dilated sinusoids in IHCA.

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CHAPTER 5

Ductular reaction and clonal expansion in Inflammatory Hepatocellular Adenoma

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Manuscript in preparation

ABSTRACT

The inflammatory hepatocellular adenoma (IHCA) which is the main subtype of hepatocellular adenoma, uniquely contains inflammation and ductular structures located in portal tract like structures. The inflammation is induced by the IHCA related mutations of the IL6ST, STAT3 and GNAS genes that all result in activation of the JAK/STAT3 pathway. In a previous study we have found increased leptin receptor expression in IHCA which may be a link with the association of IHCA with obesity and also contribute to the inflammatory activity as leptin also activates the JAK/STAT3 pathway.

In the present study we hypothesized that the ductules in IHCA represent a hepatic progenitor cell (HPC) mediated regenerative reaction, induced by the inflammation. Using the HPC markers, CK19, EpCAM and Sox-9，we found HPC in the ductules and in some hepatocytes. Inflammation was strongly associated with HPC markers expression and the presence of ductules, which supports our hypothesis and also signifies that the capability of HPC mediated regeneration is preserved in this neoplastic lesion.

In the second part of the study we applied the CCO/SDH double enzyme histochemistry and subsequent sequencing of the whole mitochondrial DNA genome to further study the ductular areas. In 67% of the cases we found clonal patches of CCO-deficient cells, located in the same area as the ductules albeit in lesser quantities. These subclones were not associated with the presence of inflammation, HPC markers expression or the presence of ductules which suggests that the clonal expansion is a constitutive phenomenon in IHCA, similar with the clonal expansion described in other solid organ tumors. Despite the absence of an association with HPC markers expression, such CCO-deficient clones have been postulated as stem cell derived in the normal and cirrhotic livers. This is not only due to its location in the hepatic stem cell niche but because an accumulation of mutations is necessary to acquire such a high degree of heteroplasmy or homoplasmy to achieve a biochemically observable CCO-deficiency. These changes require such an extended cellular lifespan that can only be met by stem cells.

We concluded that the ductules and clonal expansion in IHCA are 2 phenomena of different pathogenetic background, although both representing activation of the HPC/stem cell niche.

INTRODUCTION

The inflammatory type hepatocellular adenoma (IHCA) is the main subtype of hepatocellular adenomas (HCA), representing 40-50% of all HCA. IHCA occurs frequently in overweight female patients with long term use of oral contraceptives (1-3). Unlike the other subtypes of HCA, most IHCAs contain ductular structures and inflammatory cells, located in pseudo portal tracts (PPT). The PPT are irregularly formed and randomly distributed fibrous stromal areas of varying sizes in which arteries, arterioles and inflammatory cells are located. In the periphery of these structures, at the junction of PPT-stroma and tumor hepatocytes, ductular structures are usually found. A proper bile duct is not present. Hence, the designation of *pseudo* portal tract.

Before the monoclonal nature and the mutational background of IHCA were known, the lesion was regarded as a variant focal nodular hyperplasia (FNH) (4, 5). This was due to the presence of the ductules, inflammatory cells and PPT which were recognized as features of FNH whereas HCA was considered as a neoplasm consisting of purely hepatocytic proliferation. The histologic appearance of the ductular structures, both the cytological features of the lining cholangiocytes and the architecture of the ductules are indistinguishable from the ductular reaction (DR) that is described in several liver diseases including FNH (6). For this reason the term DR is chosen in the present study to label the ductular structures in IHCA.

The presence of DR in IHCA which is a hepatocytic neoplastic lesion is remarkable because of its non-neoplastic appearance. To the best of our knowledge, the nature of DR in IHCA has not been studied before with regard to its association with the tumor hepatocytes and the association with the inflammatory component of IHCA. Based on the current insight that DR represents a hepatocytic progenitor cell (HPC) mediated reparative response it is plausible to consider that DR in IHCA represent such a response instigated by the inflammatory activity in

IHCA. This inflammatory component is intrinsic to IHCA due to its mutational background of the IL6ST, STAT3 and GNAS genes which result in an increase of an inflammatory response due to the activation of the JAK/STAT3 pathway (7-9). Additionally, the inflammatory state may also be stimulated by the association of IHCA with obesity which is known to induce and maintain an inflammatory status, partly due to the activation of leptin. In our previous study an increased expression of leptin receptor has been found in IHCA (10).

To investigate the nature of DR in IHCA and test the hypothesis whether DR may represent a regenerative response, we first examined the incidence of DR in IHCA and studied the HPC features using a set of immunohistochemical markers of progenitor cells such as CK19, epithelial cell adhesion molecule (EpCAM) and sex determining region Y-box 9 (Sox-9). Secondly, we studied the clonal pattern of IHCA based on the mitochondrial DNA (mtDNA) mutation pattern. Several studies on cell lineage analysis have applied mtDNA mutation pattern to locate the stem cell niche and visualize the expansion of stem cell derived clones in normal and diseased organs (11-13). Basically this technique identifies cellular areas on the tissue level that contain mtDNA mutation by the absence of the enzyme cytochrome-*c*-oxidase (CCO) which is encoded by mtDNA. Using this technique, Fellous et al and De Alwis et al have identified clonal expansion originating from periportally located clonal proliferative units in the normal human liver that gave rise to large populations of hepatocytes (11, 14). Lin et al applied the same technique and established that hepatocytes in the regenerative nodules of a cirrhotic liver are monoclonal cell populations that share a common cell of origin with the surrounding DR (13). These observations confirm that ductules and DR represent the hepatic stem cell niche in the normal human liver and diseased ones respectively.

PATIENTS AND METHODS

Patients

Samples from 24 IHCA patients, all females, were included in this study (median age 29.5 years, ± 9 years). Both frozen and paraffin sections of each case were selected after being immunohistologically subtyped as IHCA based on diffuse CRP and/or SAA positivity according to the established international criteria (3). Frozen samples of non-lesional areas taken at least 3 cm from the tumor were available in 19 cases.

Histology

Paraffin slides were stained with hematoxylin-eosin and scored for DR (present or absent) and inflammation (scored as absent, mild, moderate or severe).

Immunohistochemistry

Four micron thick paraffin slides were deparaffinized and endogenous peroxidase was blocked with 0.3% H_2O_2 for 30 min. The methods of antigen retrieval and the dilutions of antibodies were summarized in Table 1. Slides were incubated with the primary antibodies for 1 hour, followed by second antibodies in Envision AEC (3-amino-9thyl-carbazole, Dako, Danmark) or DAB (3,3'-diaminobenzidine, Dako, Danmark) staining kit for 30 min at room temperature, then colour development with AEC or DAB for 10 min and mounted after hematoxylin nuclear counter staining.

Double labeling was performed for Sox-9 and HepPar1 using a similar procedure as described above except that Tris-buffered saline (TBS) was used as rinse buffer and two cocktail antibodies. The primary cocktail consisted of Sox-9 (rabbit anti-human polyclonal, 1:1000) and HepPar1 (mouse anti-human monoclonal, 1:150), and the second step cocktail contained a mix of goat anti-rabbit-horseradish peroxidise conjugated antibody and goat anti-mouse-alkaline phosphatase conjugated antibody in TBS. The staining signal was visualized by application of DAB for 10 min and 30 min of fast red substrate (50 ml 0.1M Tris-HCL pH 8.2 with 10 mg Naphtol AS-MX, 50 mg Fast red and 12 mg Levamisol;

Sigma-Aldrich, St. Louis, MO, USA), finalized by mounting with Kaiser's glycerol gelatin.

Table1 Antibodies used in immunohistochemistry

Antibody	Dilution	Company	Code	Antigen retrieval
CK19 (Mouse anti-human monoclonal)	1:100	Dako	RCK108	30min 0.1% protease
EpCAM (Mouse anti-human monoclonal)	1:1500	Merck Chemicals	OP187	30min 0.1% protease
Sox-9 (Rabbit anti-human polyclonal)	1:1000	Millipore	AB5535	15min microwave pH8.0 EDTA
HepPar 1 (Mouse anti-human monoclonal)	1:150	Dako	OCH1E5	15min microwave pH8.0 EDTA
Envision+System-HRP Mouse (DAB)	Ready to use	Dako	K4006	-
Envision+System-HRP Rabbit (AEC)	Ready to use	Dako	K4009	-
Polyclonal goat anti-rabbit immunoglobulins/HRP	1:100	Dako	-	-
Polyclonal goat anti-mouse immunoglobulins/AP	1:100	Dako	-	-

Double enzyme histochemistry

All tumor samples were analyzed and additionally, 19 available samples of non-lesional liver parts of these tumors were also included. Ten micron thick frozen sections were applied for the double enzyme histochemistry, of cytochrome *c* oxidase (CCO) encoded by mtDNA, and succinate dehydrogenase (SDH) encoded by nuclear DNA. Consecutive CCO and SDH staining could detect the deficiencies of CCO. CCO negative SDH positive areas (CCO-/SDH+) will appear blue representing areas with mtDNA mutation lacking CCO activity. Brown areas contain CCO+/SDH+ cells without mtDNA mutation (12, 15).

Air-dried frozen sections were incubated for 90 min at 37˚C with CCO medium containing 100 μM cytochrome c, 4 mM DAB, and 20 μg/ml catalase (Sigma-Aldrich, St. Louis, MO, USA) in 0.2 M pH 7.0 phosphate buffer. After washing with phosphate-buffered saline (PBS) three times, the sections were incubated with SDH medium including 130 mM sodium succinate (BDH Chemicals, England), 200 μM phenazine methosulphate, 1 mM sodium azide, 1.2 mM nitroblue tetrazolium (Sigma-Aldrich, St. Louis, MO, USA) in 0.2 M phosphate buffer pH 7.0 for 20 min at 37˚C. Sections were washed with PBS again for three times, dehydrated in a grade ethanol series and mounted with permanent mounting medium.

Laser Microdissection (LMD) and isolation of DNA

Sixteen micron frozen section of two tumor samples were cut on the 1.0 PEN membrane slides (Carl Zeiss Microscopy GmbH, Göttingen, Germany) after UV irradiation and stained with two-color enzyme histochemistry as described above. After overnight drying, two CCO-/SDH+ blue areas (No.1 and No.2) and one CCO+/SDH+ brown area (No.3) of each sample were laser microdissected and collected in the adhesive caps. After centrifuging for 10 min, the tubes were added with 20 μl lysis buffer with 50 mM Tirs-HCL pH 8.5, 1 mM EDTA, 0.5 % Tween-20 and 200 μg/ml proteinase K at 65°C for 3 hours and then denatured at 95°C for 10 minutes.

Mitochondrial DNA sequencing

Primary PCR reactions

After total DNA extraction, nine pairs of primers were designed to selectively amplify the mtDNA genome sequence with 2000bp products (15). 1 µl cells lysate was consumed in each 50 µl PCR reaction containing 1× PCR buffer (Applied Biosystems, Foster City, CA), 0.2 mM dNTP (Thermo Fisher Scientific Inc, Schwerte, Germany), 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 0.6 µM primers (Integrated DNA Technologies, Coralville, IA). The amplification conditions are as follows: 94 °C for 12 minutes; 35 circles of 94 °C for 1 min, 58°C for 1 min and 72°C for 2 min; extension time 8 min.

Second PCR reactions

Another twenty-eight pairs of primers (Integrated DNA Technologies, Coralville, IA) were applied in this round PCR to amplify the mtDNA templates of 1st PCR and gained 2nd PCR products between 600-700 bp which span the whole mtDNA genome (15). All of designed forward primers were tagged with 18 nt of M13 forward sequence 5'-TGTAAAACGACGGCCAGT-3' and reverse primers were tagged with 18 nt of M13 reverse sequence 5'-CAGGAAACAGCTATGACC-3' respectively, which are convenient for sequencing for PCR amplified products.

2 µl of 1st PCR product was consumed in each 50 µl PCR reaction as described above. The amplification conditions are as follows: 94 °C for 12 minutes; 30 circles of 94 °C for 1 min, 58°C for 1 min and 72°C for 1 min; extension time 8 min.

Sequencing and mutation analysis

Nest PCR products were purified by Roche high pure PCR product purification kit (Mannheim, Germany) and sequenced via BigDye Ver 3.1 terminator cycle sequencing chemistries on an ABI Prism 3500 Genetic Analyzer (Applied biosystems, CA), followed by compared with the revised Cambridge reference sequence (16) using ApE-A Plasmid Editor to alignment the sequences

(http://biologylabs.utah.edu/jorgensen/wayned/ape/).

Statistics

Data were analyzed by PASW Statistics 18 software (SPSS, Chicago, IL, USA). The relationship between discrete variables was evaluated with chi-square test. $p<0.05$ was set as the level of significance.

RESULTS

Histology and immunohistochemistry

All of the 24 IHCA cases were diagnosed as IHCA according to the immunophenotype of overexpression of CRP and/or SAA in the tumor (Bioulac, 2009). Inflammation, present in and around the PPT areas was seen in 20/24 cases (83%), predominantly of mild grade (70%). Fourteen of 24 cases (58%) show DR based on routine HE staining (Figure 1 A). All 14 DR positive cases were concurrently present with inflammation, the 4 cases without inflammation did not contain DR, resulting in an association of DR with inflammation ($p = 0.02$).

CK19 staining identified the same number of 14 cases containing DR (Figure 1 B) while EpCAM yielded 15/24 cases (63%) and Sox-9 labelled 16/24 (67%) DR cases (Figure 1 D). Hepatocytic positive CK19 staining was observed in 9/24 cases (38%), present as isolated hepatocytes around PPT whereas EpCAM labelled clusters of hepatocytes in 18/24 cases (75%) (Figure 1 C) and Sox-9 positive hepatocytes were present in 6/24 (25%) cases (Figure 1 D).

In the Sox-9/HepPar1 double staining, Sox9+/HepPar1- cells (Figure 1 E) were present in 17/24 cases (70%), and Sox9+/HepPar1+ hepatocytes (Figure 1 F) in 5/24 cases (20%), of which 2 cases presented as diffuse Sox9 staining. The 3 other cases showed positive clusters or single hepatocytes abutting PPT.

The Sox9+/HepPar1+ hepatocytes were found tethered to Sox-9+/HepPar1- ductular cells at the PPT side and at the parenchymal side in continuity with Sox-9-/HepPar1+ tumor cells.

All but 2 cases showed expression of at least one of the three HPC makers, either on the DR or on hepatocytes. The expression of the HPC markers on either DR or hepatocyte is strongly associated with the presence of inflammation (p=0.001). The 2 cases which are totally negative for all HPC markers were 2 cases without inflammation or PPT, while, of which 1 case with CCO-deficient patch and the other case without CCO-deficient patch in double enzyme histochemistry.

Double enzyme histochemistry

Sixteen out of 24 IHCA cases (67%) show CCO-deficient blue areas (Figure 2/3 A and B), all abutting the PPT at one side. A similar pattern was seen in the 19 samples of non-lesional liver which all contain CCO-deficient areas consistently located in juxtaposition of portal tracts. Fifteen non-lesional samples of the 16 IHCA cases with CCO-deficient patches also showed CCO-deficient areas, and non-lesional sample was not available in one case. Of the 8 IHCA cases without CCO-deficient areas, 4 non-lesional samples were available and all showed CCO-deficient areas.

Among 16 cases with CCO-deficient areas in the tumor, 10 cases have more than 2 visible patches on one single section. The median number of CCO-deficient patches in the tumor is 2 (range 1-7).

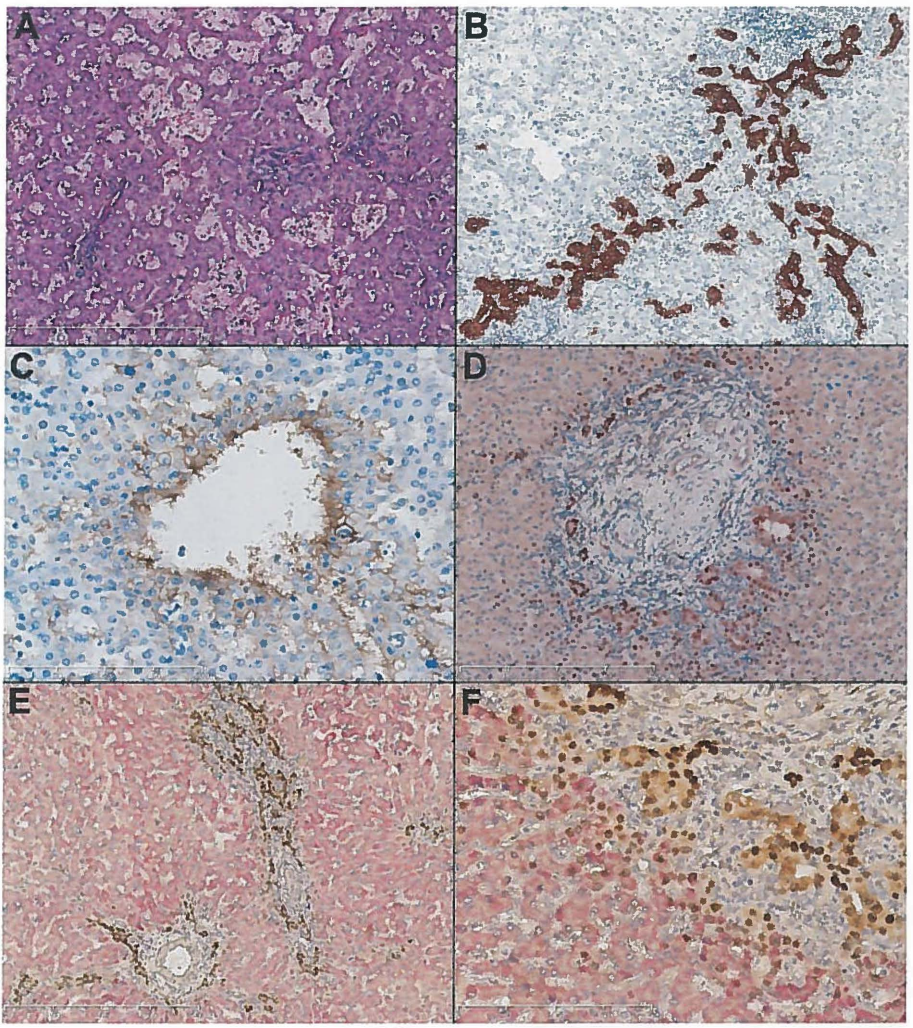

Figure 1. Immunohistochemistry staining of hepatic progenitor makers in inflammatory hepatocellular adenomes.

A: HE staining showing 2 PPT, 100×;

B: CK19 staining highlighting DR (brown DAB labeling) at the periphery of PPT, 100×;

C: EpCAM staining highlighting perivascular hepatocytes with membrane positive signal, DAB (brown), 200×;

D: Nuclear Sox-9 staining highlighting DR and some hepatocytes in continuity with DR cells AEC (red), 100×;

E: Sox-9 (nuclear, brown) and HepPar1 (cytoplasmic, pink-red) double staining. DR are Sox-9+/HepPar1-, 100×;

F: Sox-9 (nuclear, brown) and HepPar1 (cytoplasmic, pink-red) double staining. Sox-9+/HepPar1+ hepatocytes are seen abutting DR, 200×.

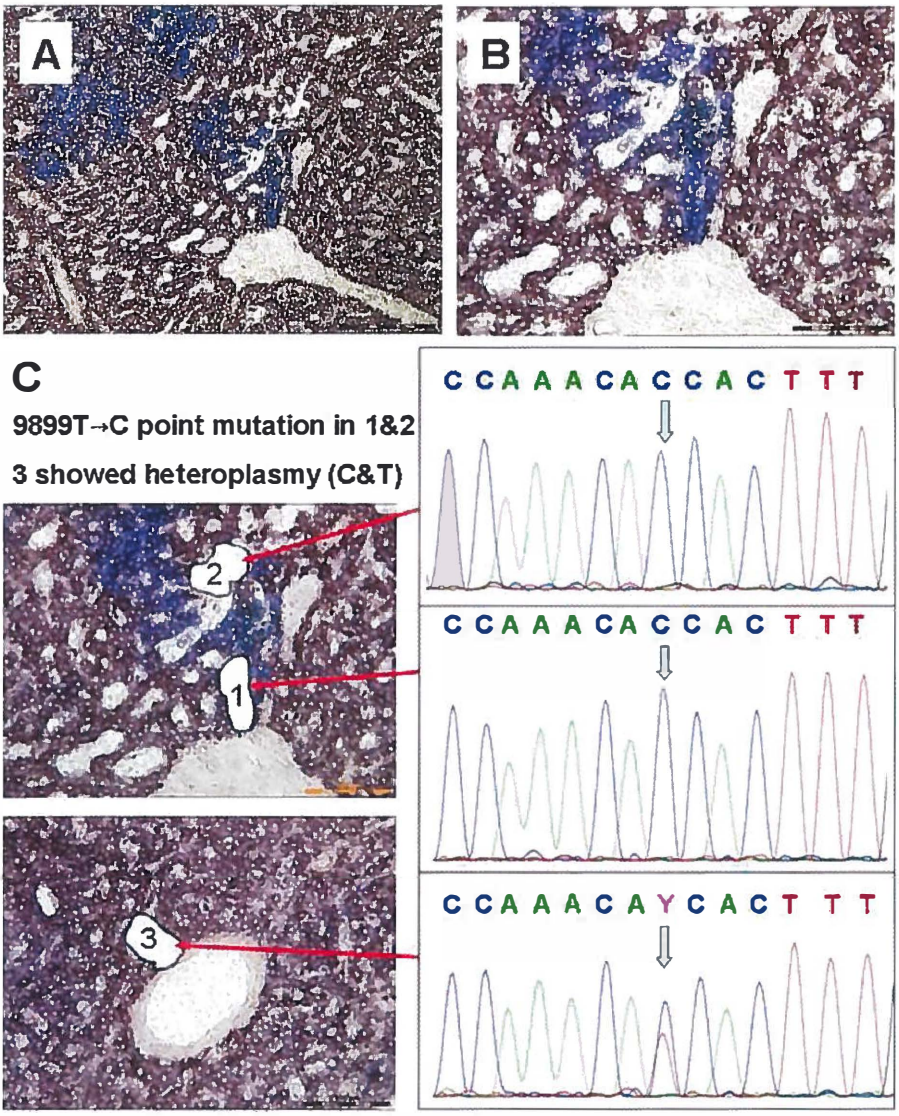

Figure 2. Double enzyme histochemistry staining (CCO/SDH) and mitochondrial DNA sequencing of CCO-deficient and CCO-positive hepatocytes in Case 1 IHCA

A (100×) and **B** (200×), CCO/SDH double enzyme histochemistry showed blue CCO-deficient areas abutting a pseudo portal tract and brown CCO-active areas.

C: Left panel, two areas (No.1 and No.2) from the same CCO-deficient patch and one area (No.3) from a CCO-active area were microdissected for mtDNA sequencing. Right panel, the m9899T→C *COIII* gene mutation was present in areas No.1 and No.2 from the CCO-deficient patch, which differs (C or T heteroplasmy) from the CCO-active No.3 area of the same tumor.

HPC features in cases with CCO-deficient patches

Nine out of 16 cases with CCO-deficient patches (56%) showed CK19+ DR, 10/16 cases (63%) contained EpCAM+ DR and 11/16 (69%) cases demonstrated Sox-9+ DR. Of note, 6/16 cases (38%) showed isolated CK19+ hepatocytes, 12/16 cases (75%) contained EpCAM+ hepatocytes mostly clustered in peri-PPT positions. Four out of 16 cases (25%) showed Sox-9+ hepatocytes, 2 cases with a diffuse hepatocytic distribution and 2 others showing peri-PPT clusters.

CCO-deficient patches were present both in the cases with DR or cases without DR. There is no association between CCO-deficient cases and inflammation. When cases were regrouped into CCO-deficient and CCO-positive cases, there is no statistical difference with regard to the presence of HPC markers expression in DR or hepatocyte.

Mitochondrial DNA mutation analysis of LMD samples

LMD was performed on two IHCA samples. Figure 2 A and B show the enzyme histochemical staining of Case 1 before LMD, in which blue CCO-deficient patches are visible. Two areas (No.1 and No.2) from a single CCO-deficient patch abutting a PPT and one area (No.3) from a CCO-positive area were captured for sequencing, followed by comparing with the original sequence of mtDNA. We found an identical m9899T→C *COIII* gene mutation in areas No. 1 and No.2 of the same CCO-deficient patch and a different pattern of heteroplasmy in No. 3 area from the CCO-positive area (Figure 2.C). The same procedure was applied for the second case (Figure 3) and the whole mutated positions were summarized in Table 2. Fourteen positions with base mutations were found in Case 1, and 2 positions of base mutations in Case 2. Among these 16 positions with base mutations, 6 positions result in amino acid mutations. In Case 2, nucleotide position 9384 G mutated to A in both area No.1 and No.2 but not in area No.3 (Figure 3 C). In both LMD cases, the base mutations of the No.1 and No.2 areas derived from one single CCO-deficient patch were consistent, and were different from the No.3 area from the CCO-positive area and the original mtDNA sequence.

Table 2 Mitochondrial DNA mutation

Gene	Nucleotid Position	rCRS*	Case 1-1 (CCO-/SDH+)	Case 1-2 (CCO-/SDH+)	Case 1-3 (CCO+/SDH+)	Case 2-1 (CCO-/SDH+)	Case 2-2 (CCO-/SDH+)	Case 2-3 (CCO+/SDH+)	Amino Acid Change
D-Loop	186	C				T	T	C	
12S rRNA	709	G	A	A	R				rRNA
ND1	3308	T	G	G	K				M-R*
ND1	4216	T	C	C	Y				Y-H
ND2	4917	A	G	G	R				N-D
CO III	9384	G				A	A	G	D-N
CO III	9899	T	C	C	Y				syn
ND5	13145	G	A	R	G				S-N
cyt b	15452	C	A	A	M				L-I
cyt b	15607	A	G	G	R				syn
tRNAthr	15928	G	A	A	R				tRNA
D-Loop	16086	T	C	C	Y				
D-Loop	16126	T	C	C	Y				
D-Loop	16163	A	G	G	R				
D-Loop	16186	C	T	T	Y				
D-Loop	16189	T	C	C	Y				

Nucleotide and amino acid codes are according to IUPAC nomenclature
* revised Cambridge Reference Sequence (16)

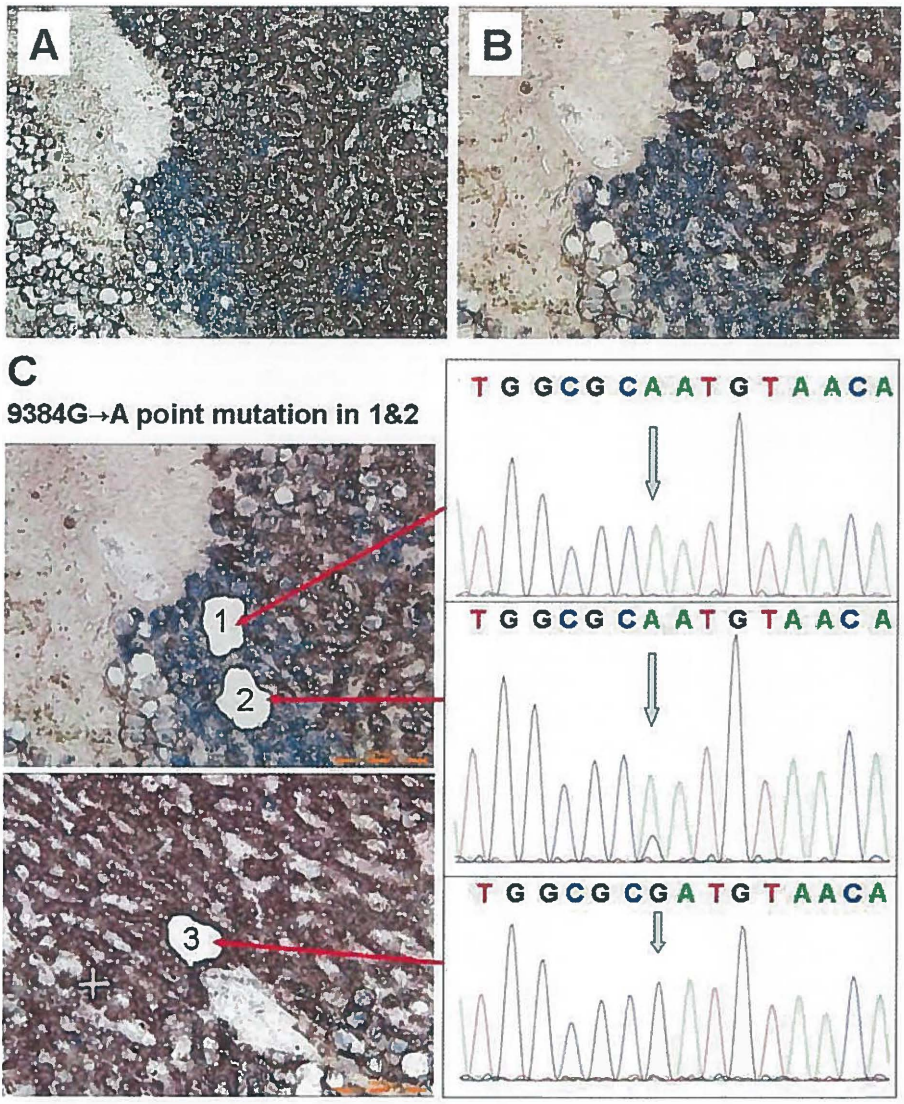

Figure 3. Double enzyme histochemistry staining (CCO/SDH) and mitochondrial DNA sequencing of CCO-deficient and CCO-positive hepatocytes in Case 2 IHCA

A (100×) and **B** (200×), CCO/SDH double enzyme histochemistry showed blue CCO-deficient areas abutting a pseudo portal tract and brown CCO-active areas.

C: Left panel, two areas (No.1 and No.2) from one CCO-deficient patch and one area (No.3) from CCO-positive area were microdissected for mtDNA sequencing. Right panel, the m9384G→A *COIII* gene mutation was present in No.1 and No.2 areas from CCO-deficient patch, which is different from G in No.3 area of CCO-positive area of the same tumor.

Serial sections for CCO-deficient patches

To determine whether CCO-deficient patches in the tumor is consistently aligned with a PPT and are not randomly distributed in the tumor parenchyma without connection to a PPT, we performed the double enzyme histochemistry staining on serial sections. This procedure showed that in subsequent slides CCO-deficient patches coalesce and finally form one single larger CCO-deficient patch that aligns with a PPT (Figure 4 A-D).

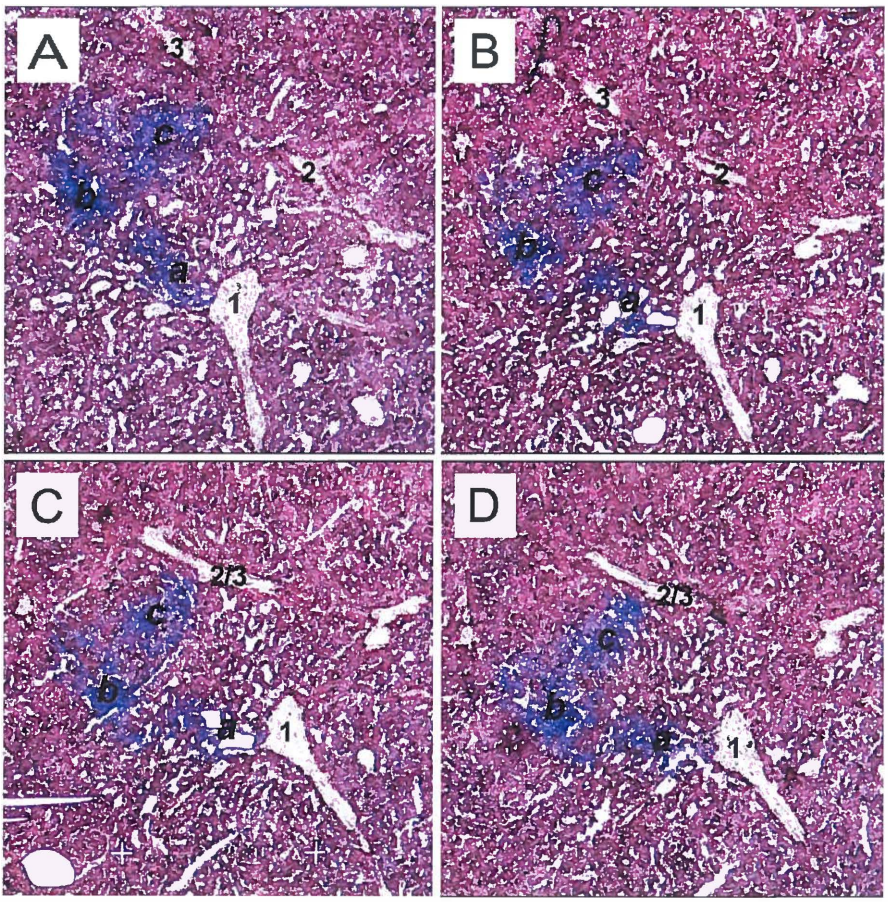

Figure 4. Serial sections with CCO-deficient patches. A to D is 16 micron distance from the previous section.

A: three separate CCO-deficient blue patches ***a***, ***b***, and ***c***, and three separate pseudo portal tracts (PPT), ***1***, ***2***, and ***3***. 100×

B, C, and **D**, CCO-deficient blue patch ***b*** and ***c*** connected with each other to form a single, larger patch, and alignment with the PPT ***2/3*** formed from formerly separated PPT ***2*** and ***3***.; the CCO-deficient blue patch ***a***, different from the patches ***b*** and ***c*** stayed connected to PPT ***1***. 100×

DISCUSSION

Ductular structures and inflammation located in or adjacent to PPT are features that are frequently found in IHCA but are usually absent in the other subtypes of HCA. We have shown that in IHCA, the presence of ductules is associated with inflammation which justifies the designation of DR to the phenomenon of ductules combined with inflammation in the PPT. Using CK19, EpCAM and Sox-9 as immunohistological HPC markers, we have identified HPC in the DR and the expression of these markers was also strongly associated with inflammation. Based on the CCO/SDH double enzyme histochemistry and subsequent DNA sequencing we have observed clonal cell populations in peri-PPT areas in the tumor and in peri-portal areas in non-lesional samples in the majority of IHCA cases. There were few clonal units per sample, both in the tumor and non-tumor samples in contrast with DR which was mostly present in all PPT in DR+ cases.

The main question to be addressed is how these 2 phenomena that share the same peri-PPT location are related with each other.

The 3 immunohistologic HPC markers that we have applied have all been recognized to identify HPC in several liver diseases as well as in normal livers (17-20). Each of these markers identify different populations of HPC and variable stages of their differentiation, underlining the diversity of HPC expansion (6, 21). In diseased livers DR has been recognized as an HPC-mediated regenerative reaction with diverse morphologic and immunophenotypic appearance depending on the type and extent of liver damage (6, 22, 23). In this study we found that both DR and HPC were strongly associated with the presence of inflammation which supports our hypothesis that the inflammatory activity in IHCA would induce an HPC mediated regenerative reaction represented by DR, in a similar way with DR that can be found in non-neoplastic diseased livers (6). The inflammation in IHCA is probably induced by the IHCA related mutations of the IL6ST, STAT3 and GNAS genes that all lead to activation of the JAK/STAT3 pathway and stimulate recruitment of inflammatory cells via CCL20 induction (7, 8). Furthermore, the association of IHCA with obesity can also contribute to the inflammatory status through the leptin/LR pathway that also activates the JAK/STAT3 pathway (10).

We have also found HPC immunophenotypes on a small number of hepatocytes as shown by the single staining of each of the HPC markers and confirmed by the double labelling of the HPC marker Sox-9 and the hepatocyte marker HepPar1. In a recent study Furuyama et al documented Sox-9 labeling of bile ducts and canals of Hering, but not hepatocytes (19). These investigators documented that during liver regeneration following CCl_4 induced injury there was proliferation of biliary ducts expressing Sox-9, including Sox-9 positive canals of Hering, and that these HPC contribute to hepatocytic replenishment as shown by lineage labeling. However, the hepatocytes were consistently Sox-9 negative (19).

In our IHCA samples, the Sox-9+ hepatocytes were found at the peri-PPT sites and occasionally a cell is seen tethered to a ductule at the PPT side while linked to a Sox-9 negative hepatocyte at the parenchymal side. While Sox-9 expression confirms the progenitor cell status of these cells, HepPar1 expression denotes a hepatocytic differentiation. This immunophenotype and the unique location of these cells suggest that these hepatocytes may represent the peribiliary hepatocytes (24). These cells are thought to represent HPC derived specialized hepatocytes which provide the reconnection of hepatocytes with the smallest units of the biliary tree, in order to restore parenchymal function by providing biliary drainage (24, 25). These findings were based on studies on diseased livers but not in neoplastic growth. The question arises whether this type of restoration and thus these linkage cells are needed in an adenomatous growth. The presence of biliary excretion function in HCA is shown by the application of gadolinium, which is a liver specific contrast agent in MRI investigations. Uptake of this agent occurs via the transporter QATP1B1 and B3, present at the sinusoidal membrane while biliary excretion takes place via MRP2, the multidrug resistance-associated proteins present at the canalicular membrane (26). The presence of several types of hepatic transporters in HCA has also been documented, in which HCA hepatocytes showed the expression of several transporters involved in bile secretion, such as BSEP, MDR1, MDR3, MRP2, MRP3 and focally OATP2/8 (27). These findings suggest that a proper reconnection between hepatocytes and the biliary tree is necessary in HCA and the fact that cholestasis is not a frequent phenomenon in HCA implies that the restoration of the hepatobiliary linkage can take place

adequately. Furthermore, our results also signify that the HPC mediated response to injury is still preserved in this neoplastic growth.

In the second part of our study, using the CCO/SDH double enzyme histochemistry and subsequent mtDNA sequencing, we have found clonal cell populations at the periphery of PPT in the majority of IHCA samples. Seemingly randomly distributed CCO-deficient patches appeared to be aligned with a PPT after serial sectioning which corroborates the notion that the mtDNA mutated clonal cell populations are consistently located at the peri-PPT area. This pattern is identical to the findings described in normal livers based on which it has been postulated that these populations represent the stem cell niche that can give rise to large numbers of hepatocytes, probably as part of the normal process of hepatocyte renewal (11, 14). Such CCO-deficient patches were also found in all available samples of non-lesional tissue, taken distant from the tumor and also present in 50% of those cases which did not show CCO-deficient areas in the tumor itself. These results indicate that the presence of periportal or peri-PPT clonal cell populations is a common feature in IHCA, both intratumoral and in the non-lesional liver.

The identical location of the clonal cell populations and HPC containing DR at the peri-PPT areas in our IHCA samples indicate that these peri-PPT areas represent a stem-cell niche that can generate clonal cell populations that carry a different genetic make-up compared with the surrounding hepatocytes. The fact that the presence of these CCO-deficient clones is not associated with the presence or absence of inflammation nor with DR, indicates a constitutive process of clonal expansion. However, the inflammatory component of IHCA did show an association with HPC and with DR, which signifies an induction of an HPC mediated regenerative response by the inflammatory activity. Our findings indicate that the peri-PPT areas in IHCA contain 2 HPC related phenomena, a constitutive clonal expansion and a regenerative response.

Although there is no statistical correlation between the presence of the CCO-deficient patches with the HPC immunophenotype, the peri-PPT clonal expansion should be assumed to be stem cell related. As has been discussed by Fellous et al, observable mtDNA mutational phenotype requires homoplasmy or a high degree of

heteroplasmy which can only be reached by accumulation of mitochondrial mutations which takes many years following a stochastic expansion. This process is only suitable for stem cells due to their unique sufficient life span (11). Thus, a CCO-deficient clonal patch which is enzyme-histochemically visible on the tissue level represents the progeny of a stem cell. The difference in extent of HPC expansion recognizable as DR and present in practically all PPT in an IHCA sample is in contrast with the rare occurrence of CCO-deficient patches in one or only a few PPT area. This pattern supports the idea that we are dealing with 2 phenomena of different pathogenetic backgrounds. HPC mediated DR is most probably reactive to inflammation and cellular injury whereas the clonal expansion is a constitutive process.

IHCA is regarded as a monoclonal and neoplastic lesion in contrast with FNH which is a polyclonal and hyperplastic condition (5). Our findings of the presence of intratumoral subclonal cell populations do not argue against the neoplastic character of IHCA. Recent studies have shown that neoplastic growth includes a process of clonal evolution of tumor cells that generates subclones as progeny of sequential mutations during tumor progression (28-30). Although most studies are related with malignant tumors, the presence of subclones within colorectal adenomatous growth has recently been reported, based on mtDNA mutations and methylation diversity (31). Similarly, stem-cell derived clonal units that replenish the prostatic epithelium have been documented in normal prostates and also in diseased conditions including benign and malignant neoplastic prostatic growth (32).

According to current insights in tumor biology, the generation of subclones in neoplastic lesions is part of the process of clonal evolution which is regarded as an adaptive mechanism of the neoplastic clones, in parallel with the Darwinian principle of evolution (29). Random mutations in clonal expansions combined with natural selection of the variants most adequately adapted to the tissue ecosystems will allow the neoplastic population to achieve and maintain uncontrolled growth.

To the best of our knowledge, the presence of subclonal populations in a HCA variant has not been previously described and further studies are obviously

necessary to further characterize the potentials of these subclones, especially with regard to carcinogenesis.

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CHAPTER 6

Summary

Discussion and Future Perspectives

SUMMARY

Hepatocellular adenoma (HCA) is the second most common hepatocellular benign tumor in the liver next to focal nodular hyperplasia. HCA occurs predominantly in reproductive females with long term usage of oral contraceptives. Inflammatory hepatocellular adenoma (IHCA) is the most frequently occurring subtype of HCA and can be classified by its genotype. Sixty percent of IHCA contains a somatic mutation of interleukin-6 signal transducer (IL6ST), 8-12% a mutation of signal transducer and activator of transcription-3 (STAT3) mutation and 5% a mutation of G-protein alpha stimulatory subunit (GNAS), which all can lead to activation of STAT3 without IL-6 binding. Regardless of the mutation types, IHCA show increased C-reactive protein (CRP) and/or serum amyloid A (SAA) expression which enables diagnosing IHCA by immunohistology. Morphologically IHCA is characterized by the presence of inflammatory cells, sinusoidal dilation, single arteries and ductular reaction (DR) located around pseudo portal tracts (PPT). An important epidemiological fact is the association between IHCA and females with a high body mass index (BMI).

In this thesis, we aimed to investigate whether a link exists between obesity with IHCA and gain insight in the microenvironment in which IHCA develops. We studied the non-lesional liver parts in IHCA patients; the status of adipokines receptors related with obesity in tumor and non-tumorous livers; the influences of leptin on endothelial cells activation with leukocytes adhesion in an *in vitro* study and the nature of DR in IHCA.

In **chapter 2**, we investigated the histological and immunological changes of non-lesional liver of 32 resected samples of IHCA patients and found that abnormal changes were present both in the liver adjacent to the tumor and distant liver samples. Mild to moderate steatosis were present in high frequencies which is in line with the high median value of BMI (32.55 ± 4.9) of the studied patients. Dilated sinusoids and single arteries were regularly seen even in the distant liver samples. An important finding was the presence of minute CRP positive foci in circa 50% of the cases demonstrating small IHCA foci mostly present in IHCA patients with multiple nodules. This suggests that the non-lesional parts of IHCA-livers harbor

changes that may potentially stimulate growth of new adenomas. These findings may influence the follow-up management, especially since it is known that obesity may also stimulate malignant growth. Based on the hypothesis that obesity might contribute to IHCA tumorigenesis due to secretion of adipokines by visceral adipose tissue and direct hepatic targeting via the portal vein, we designed the studies in **chapter 3**.

In this chapter, we investigated the expression status of leptin receptor (LR) and adiponectin receptor 2 (AdiR2) in 25 cases of IHCA and found that the LR gene was upregulated in the tumor compared to its non-lesional counterpart and normal liver controls. The AdiR2 gene was significantly down-regulated in the tumor. Activation of the leptin-LR pathway and its downstream signaling effects was shown by the presence of nuclear p-STAT3 expression and an increase of STAT3 gene expression in the tumor. LR was mainly expressed on sinusoidal endothelial cells (SEC) and nuclear p-STAT3 expression was observed not only on the nuclei of hepatocytes but also on the nuclei of SEC. In an *in vitro* study, leptin stimulation upregulated IL-6 gene expression in both human umbilical vein endothelial cells (HUVEC) and human hepatic sinusoidal endothelial cells (hHSEC). These data suggest that leptin may contribute to the inflammation and proliferation status of IHCA and represent the first results that link obesity with IHCA.

Based on the findings that mRNA levels of LR were upregulated in IHCA and that LR was expressed on SEC, we assumed that in IHCA leptin might play a proinflammatory role via binding to LR on SEC and contribute to the inflammatory phenotype of IHCA. Hence, in **chapter 4** we focused on the proinflammatory influences of leptin-LR activation in HUVEC and hHSEC. Both types of endothelial cells expressed LR, and STAT3 was rapidly activated upon leptin stimulation. Simultaneously, the negative feedback loop of STAT3 activation was also activated as shown by the up-regulation of mRNA levels of suppressor of cytokine signaling 3 (SOCS3). mRNA levels of intercellular adhesion molecule-1 (ICAM-1) in HUVEC and hHSEC were upregulated to a minor extent and there was also increased ICAM-1 protein expression in HUVEC, but this did not result in an increase of HL60 leukocytes adherence. No effects of leptin on leukocyte adhesion could be observed in conjunction with TNFα induced endothelial activation.

In IHCA patients, the microenvironment is likely much more complicated than what we mimicked in **chapter 4**. For instance, besides hyperleptinemia, obese/overweight patients usually have hypoadiponectinemia and higher level of serum TNFα. Hypoadiponectinemia and lower AdiR2 gene expression in tumor will result in a reduction of protective effects and antagonization of leptin in IHCA, while serum TNFα might be the main and potent effector for leukocytes adhesion.

A continuous inflammatory state and vascular abnormalities such as dilated sinusoids in IHCA could result in hepatic parenchymal damage. In many liver diseases DR has been regarded as the hepatic progenitor cells (HPC) mediated liver regeneration and IHCA frequently contains DR which is only very rarely found in the other subtypes of HCA.

Therefore, in **chapter 5**, we hypothesized that DR in IHCA represents a regeneration activity and found DR in 58% IHCA cases while 83% cases showed mild/moderate inflammation. The inflammatory cells are located in the pseudo portal tracts which are unique structures of IHCA too, while DR is consistently found adjacent to the PPT. Immunohistological identification of HPC was performed by applying the HPC markers CK19, epithelial cell adhesion molecule (EpCAM) and sex determining region Y-box 9 (Sox9) in IHCA. Cells positive with these markers were found within DR, representing cholangiocytes with HPC features. A variable number of hepatocytes were also found to express these markers although in much lesser quantities than ductular cells. Of note, the presence of inflammation is strongly associated with the presence of DR and the expression of the HPC markers. We further investigated the clonal status of the peri-PPT areas where DR is located, using the two-color enzyme cytochrome *c* oxidase/ succinate dehydrogenase (CCO/SDH) histochemistry technique and subsequent mitochondrial DNA (mtDNA) sequencing. The latter revealed an identical mutation pattern of mtDNA in cells within a single CCO-deficient patch which differed from the surrounding CCO-positive hepatocytes. CCO-deficient patches, each representing clonal cell populations were present in 67% of IHCA. The CCO-deficient areas, indicating cells with loss of CCO production were consistently found in peri-PPT areas. Although the occurrence of these clonal CCO-deficient patches was not associated with the presence of inflammation, DR

or expression of HPC markers. Nevertheless, the clonal populations showing the mtDNA mutations are stem cell derived. The observable CCO-deficiency could only be achieved after extensive accumulations of mtDNA mutations for which an extended life span is necessary which could only be met by a stem cell.

These data demonstrate that while DR in IHCA patients represents a HPC mediated regenerative response, the clonal expansion shows a constitutive pattern. Despite the fact that both phenomena are found at the same location that can be regarded as the stem-cell niche, the 2 processes have different pathogenetic backgrounds.

Summarizing, in this thesis on the microenvironment of IHCA, we found that obesity may influence the liver in which IHCA develops and the IHCA itself, probably through release of adipokines, contributing to growth and an inflammatory phenotype. The latter may induce a HPC-mediated regeneration while the HPC/stem cell niche generates subclonal cell populations independent of the inflammatory status.

DISCUSSION AND FUTURE PERSPECTIVES

Background liver in IHCA

IHCA is the most frequently occurring subtype of HCA and is predominantly found in young women with long term use of oral contraceptives. Similar with the other HCA subtypes, IHCA develops in an otherwise non diseased liver. But, unlike the other subtypes, IHCA is frequently associated with obesity/overweight as expressed in high BMI (1, 2).

Overweight/obesity is a systemic disease, which has been documented as an increased risk factor for many diseases and/or even obesity-associated tumors. As obesity is globally increasing, non-alcoholic fatty liver disease (NAFLD) as the hepatic complication of obesity is becoming an endemic liver disease in the United States and European countries (3-5) and being recognized as another risk factor for hepatocellular carcinoma (HCC). A recent study documented 116 cases of HCC in non-cirrhotic NAFLD/non alcoholic steatohepatitis (NASH) (4) corroborating the

notion that obesity is a proven independent risk factor and "fertile soil" for NASH and HCC (6).

In the previous chapters we addressed these two significant issues of IHCA: its association with a high BMI and its occurrence in an otherwise non-diseased liver. The questions related to the first issue are whether obesity/overweight is a "fertile soil" for IHCA and how obesity influences the growth of IHCA. With regard to the second issue we investigated whether the non-lesional part of the IHCA containing liver was really normal, given the fact that obesity is a systemic disease.

In **chapter 2**, we investigated the non-lesional liver of IHCA histologically and immunohistologically including the presence of IHCA-specific features, such as CRP and/or SAA expression. We found additional CRP positive foci (less than 2 mm) demonstrating the presence of minute IHCA. As these CRP positive foci always occurred in patients with multiple IHCA, it predicts that the remnant livers of these patients may have the potential to harbor other foci of neoplastic nodular growth. The recent study by Evason et al demonstrated that IHCA with focal atypical morphological changes or clinically atypical features like male gender or female of ≥ 50 years can show β-catenin activation and/or chromosomal abnormalities, which are associated with the development of well-differentiated HCC (7). Therefore, close follow up and biopsy analysis are beneficial for the prediction of IHCA development and progression (8). As a matter of fact, HCA has not been reported in women older than 66 years (9-11). The question arises whether there is a "special" stage for malignant transformation when HCA bearing young female patients become older. Does intrinsic estrogen protect the malignant transformation from HCA when they are young? Do all HCA regress after the menopause? A long term follow up study on these patients combined with a comparative molecular study on HCC occurring in young female patients with high BMI and IHCA may give us information on the transformation from HCA to HCC.

Another finding in **chapter 2** is that steatosis (NAFLD) is present in 60-70% non-lesional liver parts in IHCA patients, which should thus be probably regarded as a "sick" liver. Based on the steatotic status of the non-lesional liver, 50-62% of steatotic tumors arise in steatotic non-lesional livers. This finding is not only in line with the high BMI features of these patients but also suggests that the tumor may

also be influenced by the systemic effects of obesity. This might enhance the tumorigenic effect in a similar way as a steatotic liver influences the development of HCC in NASH. So, what are the influences of obesity on adenomas?

Leptin and regeneration/proliferation in IHCA

Many epidemiological studies have provided associations of obesity and cancer, including HCC (12). Coexistence of obesity with chronic hepatitis B or C can increase the risk for HCC (13). Obesity can initiate HCC transformation from HCA in male patients, as reported by Farges et al (14), and the mechanisms underlying obesity and tumorigenesis are not well understood. The potential mechanisms involve insulin resistance, adipokines and low-grade inflammation as described in the **Introduction** part, based on the assumption that adipose tissue in obese individuals may influence tumorigenesis in a paracrine and systemic manner. The liver can be easily targeted in a paracrine manner by cytokines generated by the visceral adipose tissue via the portal vein. Leptin and adiponectin are the main adipokines derived from subabdominal adipose tissue and visceral adipose tissue (15). It is established that overweight/obese patients have hyperleptinemia and hypoadiponectinemia, a favorable condition for tumor growth as leptin has the potential to stimulate tumor growth while the protective growth inhibitory effect of adiponectin is decreased (15-18).

In **chapter 3**, we found that gene expression of LR is higher in the tumor compared to the corresponding non-tumorous liver while the gene expression of AdiR2 is lower in the tumor. Leptin-LR binding can activate the Janus kinase 2 (JAK2)/STAT3 pathway. The STAT3 pathway could lead to transcription of genes such as cyclin D1, Bcl-xl, Mcl-1, vascular endothelial growth factor, and survivin, that mediate proliferation, angiogenesis and cell-survival related with tumorigenesis (19).

In the liver, the effect of leptin on liver growth has been studied in liver regeneration. Leclercq et al found that the cause of impaired regeneration following toxic liver injury by carbon tetrachloride (CCl_4) in *ob/ob* mice is leptin-deficiency. This deficiency can be corrected by restoring TNF and IL-6 release, and expression of cyclin D1 catalyzing the transition from G1 to S phase of the cell

cycle, induced by leptin replacement (20). One recent study by Sydor et al demonstrated that liver regeneration after 70% partial hepatectomy is enhanced in western diet fed mice by elevated expression of hepatocyte growth factor and extracellular signal-regulated kinases (ERK) signaling with increased leptin levels in serum (21). Thus, leptin may stimulate hepatic regeneration by activating ERK. Similarly, Chen et al showed that leptin could promote proliferation in HCC cell lines by upregulation of cyclin D1 and downregulation of pro-apoptotic Bax by JAK2 linked pathway (22). Another study by Saxena et al also mentioned that leptin could increase growth, invasiveness, and migration of HCC cell lines via the JAK/STAT-PI3K/AKT-ERK axis (23). Leptin has also been found to upregulate human telomerase reverse transcriptase (TERT) involved in tumorigenesis via the JAK2/STAT3 pathway in HCC (24). Of note, a TERT promoter mutation has been found in malignant transformation of HCA (25), which suggests that leptin may take part in the malignant transformation in IHCA as well. Apart from HCC, more studies reported that leptin is involved in growth of several other tumor types via the JAK2 linked pathways, including breast cancer (26-28), gastric tumors (29, 30), colon cancer (31, 32) and lung cancer (33). Altogether, leptin effects are mediated by a JAK2 initiated cascade signaling which upregulates cell cycle proteins to accelerate cell cycle progression to stimulate cell proliferation, and downregulate anti-apoptosis proteins to prevent cell from apoptosis.

On the protein level, hepatocytic expression of LR by immunohistochemistry is rarely described. We observed LR expression (**chapter 3**) in IHCA and non-lesional liver on SEC, hepatic stellate cells (HSC) and cholangiocytes of bile duct and less obvious in ductules. Hepatocytic expression was not seen. Induction of hepatocytic LR expression has been shown in immortalized human hepatocytes with hepatitis C virus core genomic region transfection (34). Ribatti et al found a membrane-lining/cytoplasmic staining of LR in a few HCC cells using immunohistochemistry on formalin fixed paraffin embedded tissue, but diffuse and strong expression of LR located on SEC which is related with angiogenesis in HCC (35). HCC cell lines have been reported to express both the short and long forms of LR (22, 23). In a limited number of animal studies, reports on the expression of the two LR forms on murine hepatocytes are not consistent. Schroyen et al mentioned

that isolated hepatocyte was the only cell type expressing both forms of LR in Balb/C mice, in contrast to the findings of Zhao et al who reported that isolated hepatocytes only express the short form of LR in Harlan Sprague-Dawley rat (36, 37).

Nevertheless, the difficulties in showing hepatocytic LR expression on the protein level do not imply its absence. It is well-known that liver plays an important role in metabolism and exerts many functions including glucose metabolism, fatty acid oxidation and protein synthesis. Leptin regulates food intake and energy expenditure mainly via the central nervous system but also exerts direct effects on the liver (15). Studies by Raman et al and De Souza et al demonstrated that reduction of the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase is induced by leptin in isolated rat hepatocytes and obese mice, respectively (38, 39). Moreover, Nakamuta et al investigated the fatty acid metabolism between obese and non-obese NAFLD patients and found that peroxisome proliferator-activated receptor α, a major transcription activator of genes involved in mitochondrial β-oxidation, is downregulated in obese patients due to leptin resistance (40). Decreased mitochondrial β-oxidation led to steatosis in hepatocytes and caused liver damage in these patients. These functional studies on leptin corroborate the notion of hepatocytic LR expression, although it is currently beyond most immunohistologic detection methods to visualize this expression.

It is clear that future studies on the influence of leptin on hepatic tumor growth should include the cellular location of LR in the liver and the influence of leptin stimulation on the several liver cell constituents. It is conceivable that SEC, HSC and cholangiocytes may all produce different factors following leptin stimulation that may stimulate the growth of hepatocytes. In this regard, growth factors analysis of the conditioned medium derived from leptin incubated SEC, HSC and cholangiocytes can be a first step.

Leptin and inflammation in IHCA

The presence of inflammatory cells is another expression of inflammation in IHCA, besides the overexpression of the acute phase proteins CRP and/or SAA. Although

the inflammatory infiltrates are polymorphous, T lymphocytes of the CD4 and CD8 subtypes are predominant, due to the robust upregulation of CC chemokine ligand 20 (CCL20) gene expression induced by IL6ST and STAT3 mutation in IHCA (41, 42). Apart from these mutations, obesity may also contribute to inflammation in the tumor as it is a chronic inflammatory state created by the increased levels of CRP and IL-6, and adipokines including the proinflammatory cytokines such as leptin and TNFα (12).

Based on the findings in **chapter 3** of upregulation of LR in the tumor and its localization on SEC, **chapter 4** addressed the issue that leptin might play a proinflammatory role in IHCA via activation of LR on SEC. In this *in vitro* study, we showed that HUVEC and hHSEC both expressed the two forms of LR, and that STAT3 was rapidly activated upon leptin stimulation. In accordance, mRNA of SOCS3 was augmented upon short term exposure to leptin. Leptin exposure resulted in upregulation of mRNA and protein levels of ICAM-1, though to a minor extent, and did not affect leukocyte-endothelial adhesion.

The chronic low grade inflammation caused by overweight/obesity is systemic, but the inflammatory cells infiltration is present predominantly in the tumor. This discrepancy may be explained by the higher local CCL20 expression in the tumor as noted earlier. Furthermore, ductular reaction is frequently present in tumor. One recent study by Oo et al found that CCL20 was secreted by cholangiocytes in human livers containing inflammation, which is important to position Th17 cell in portal tracts in liver diseases (43). Hence, CCL20 may also be produced by cholangiocytes of the DR in IHCA and increase the recruitment of inflammatory cells around the pseudo portal tracts in the tumor where the DR is also located. Moreover, both gene and protein expression of functional LR is upregulated in the tumor (**chapter 3**).

Many immune cells express LR, of which the activity can be regulated by leptin, including monocytes, lymphocytes and macrophages (44-46). As studied by Guzik et al T cell activation markers (e.g., IL2 receptor) on the surface of CD4+ T cells were robustly increased in mice following infusion of leptin for 14 days (47). Leptin could stimulate the release of proinflammatory cytokines like TNFα by circulating monocytes (48, 49), which will induce an inflammatory phenotype on

endothelial cells. As a response to leptin, LR expressing EC will increase the production of reactive oxygen species and result in upregulation of endothelial cell adhesion molecule favoring recruitment of immune cells (50). Leptin has been found to modestly increase mRNA expression of LR and ICAM-1 in isolated rat aortic endothelium (51), which is in line with our findings of minor upregulation of ICAM-1 in HUVEC and hHSEC following leptin stimulation (**chapter 4)**.

Minor regulation of ICAM-1 and no effects on leukocyte adhesion induced by leptin may suggest the following questions. The key cytokine for adhesion induction might be TNFα, as described in Kralisch's study that a minute concentration (0.25 ng/ml) of TNFα could induce monocyte adhesion to HUVEC rather than 1000 ng/ml leptin (52). Increased serum levels of TNFα exists in overweight patients (53). Furthermore, dysfunctional SOCS3 inhibition might result in continuously activated leptin-LR pathway. SOCS3, as the negative feedback for leptin-LR pathway (54), was rapidly upregulated in HUVEC exposed to leptin (**chapter 4**). However, in **chapter 3**, we detected SOCS3 gene expression in tumor and non-tumor tissues and did not find any difference between the two locations. Of note, in the mostly obese IHCA patients, LR is over-expressed in the tumor combined with hyperleptinemia. Hence, when SOCS3 inhibition fails in the tumor, the leptin-LR binding in the tumor can be activated continuously which might upregulate ICAM-1 robustly by SEC. Moreover, the presence of lower levels of serum adiponectin in obese patient and less AdiR2 gene expression in tumor (**chapter 3**) might amplify the influences of leptin. Adiponectin plays an anti-inflammatory role in peripheral tissue. Adiponectin could decreases T cell activation and proliferation, induce the production of anti-inflammatory cytokines such as IL-10, and enhance nitric oxide production by endothelial cells to inhibit the production of endothelial cell adhesion molecules (47, 50).

Future studies that focus on the inflammatory part of IHCA may start with analysis of the serum concentration of cytokines of IHCA patients, e.g. IL-6, TNFα, leptin, adiponectin and resistin. Further study could include knocking out SOCS3 gene expression in endothelial cells or leptin stimulation on leukocytes instead of endothelial cells, and measure the effects on the leukocyte adhesion induced by leptin.

Ductular reaction (DR) in IHCA

Before the genetic background of IHCA was identified, IHCA had been recognized as telangiectatic focal nodular hyperplasia, due to the presence of DR, while HCA was considered as a neoplasm of purely hepatocytic proliferation (55). DR occurs ubiquitously in acute or chronic liver diseases in which there is extensive liver damage and/or dysfunctional proliferation capacity (56). The presence of DR in IHCA is remarkable and leads to three questions: its origin, its role in IHCA and whether newly derived hepatocytes alter the monoclonal nature of IHCA.

Where does DR come from?

Apart from the fact that steatosis was found in 60-70% of the background livers of the studied IHCA in this thesis (**chapter 2**), the mutational background of IHCA may continuously lead to JAK/STAT3 pathway activation which subsequently lead to an inflammatory status in the tumor while leptin/LR activation may also contribute into STAT3 sensitization (**chapters 3 and 4**). This continuous inflammatory status may initiate a regenerative response, including HPC activation, which manifests as DR. In **chapter 5** we have shown that in IHCA, inflammation is strongly associated with the presence of DR and the expression of the HPC markers. These findings support our hypothesis and also indicate that in this neoplastic lesion the function of HPC-mediated regeneration is preserved.

What is the role of DR in IHCA?

Obviously, the basic function of HPC is replenishment of hepatocytes. Both in human and mice, the degree of DR is dependent on the presence of liver damage (57, 58). Many studies have documented the renewal of hepatocytes from DR using several HPC markers, e.g. EpCAM which is absent in mature hepatocytes (59, 60). Zhang et al observed that EpCAM is expressed on a small group of cells connecting to the canals of Hering in adult livers (58) while Yoon et al found that EpCAM positive hepatocytes are newly derived from differentiation of EpCAM positive HPC in DR (61). Furthermore, Furuyama et al studied the cell lineage by tracing Sox-9 positive cells in adult mouse following CCl_4 administration and suggested that hepatocyte differentiation from Sox-9 positive precursors in DR

exists in the regeneration model after hepatic injury (62). These data strongly suggest that DR is a source of new hepatocytes in chronic liver disease in human or in animal models of hepatic regeneration. Although the ultimate proof that DR also contributes to the replenishment of hepatocytes in IHCA requires lineage tracing methods, the strong association of inflammation with DR and with the expression of diverse HPC markers supports the notion that the HPC-mediated regenerative response is active in IHCA.

In **chapter 5**, we did not only find DR expressing CK19, EpCAM and Sox-9 but also small hepatocytes expressing these HPC markers, located in peri-PPT areas. A few Sox-9 expressing hepatocytes are found in a unique position next to a ductular cholangiocyte on the one side and on the other side in continuity with another Sox-9 negative hepatocyte. This unique arrangement suggests that these cells may represent a specialized group of cells, the peribiliary hepatocytes. Theise et al. postulated that during liver regeneration these cells provide the reconnection of hepatocytes with the smallest units of the biliary tree, in order to restore parenchymal function by providing biliary drainage (63, 64). As discussed in **chapter 5**, the restoration of the biliary drainage in IHCA seems to be necessary as the IHCA hepatocytes are equipped to perform this function as has been shown by the presence of most of the transport proteins involved in biliary secretion (65). That the restoration process can be accomplished adequately is supported by the fact that cholestasis is not a frequent feature in IHCA. Moreover, the uptake and excretion of gadolinium, a liver specific MRI-contrast agent depends on the biliary salt transporters and these processes are also functional in HCA.

Do the newly derived hepatocytes change the "monoclonal" feature of IHCA?

Although neoplastic tumors are generally regarded to have a monoclonal origin, there is evidence that some tumors are of a polyclonal nature, e.g. tumors of the colon (66, 67). A mice study by Thliveris et al demonstrated that carcinogen-induced intestinal tumors are derived from multiple progenitors and the same group of investigators has shown formation of a polyclonal population induced by an initial progenitor recruitment of neighbouring cells with facilitated transformation within a short distance (68, 69). Furthermore, one recent study by Gaisa et al found

that a prostate cancer appears to be of polyclonal origin due to the fact that only parts of the cancer were related with the corresponding prostatic intraepithelial neoplasia lesion (70).

Apart from the HPC-mediated regenerative response in IHCA represented by DR, we also found CCO-deficient areas in 67% of IHCA cases (**chapter 5**). In the subsequent mtDNA sequencing, these CCO-deficient patches proved to be clonal cell populations. Their presence was not associated with inflammation, DR or HPC expression. This suggests that this clonal expansion is a constitutive process in IHCA, in a similar way as has been described in colorectal adenomas and benign prostatic diseases (70, 71). Thus, we showed that IHCA contains subclonal populations of hepatocytes.

Despite the absence of statistical correlation between the presence of the CCO-deficient patches with the HPC immunophenotype, the clonal expansion is stem cell related. Such enzyme-histochemically observable mtDNA mutational phenotype requires homoplasmy or a high degree of heteroplasmy. These changes require accumulation of mitochondrial mutations which takes many years following a stochastic expansion. Only stem cells with their unique, sufficient life span can meet such requirements (72). Thus, a CCO-deficient clonal patch represents the progeny of a stem cell.

The difference in extent of HPC expansion recognizable as DR and present in practically all PPT in an IHCA sample is in contrast with the rare occurrence of CCO-deficient patches in one or only a few PPT areas. This pattern supports the idea that we are dealing with 2 phenomena of different pathogenetic backgrounds. HPC mediated DR is most probably reactive to inflammation and cellular injury in IHCA whereas the clonal expansion is a constitutive process in a neoplastic lesion.

In order to confirm our observations in IHCA, in future studies we can investigate the relation of HPC expansion with and without inflammatory conditions or other types of cellular damage, e.g. hypoxia due to haemorrhage which is a frequent finding in HCA. To confirm the constitutive character of clonal expansion, the presence of subclones should be studied in other types of HCA and other techniques can also be applied such as polymorphic variant of X-linked genes, detection of microsatellite slippage (73).

In this thesis, we focused on the microenvironment in which IHCA develops. Based on the frequent presence of steatosis and the findings of foci of microadenomas in the non-lesional liver part, the liver in which IHCA develops can be regarded as "sick" livers in which other foci of IHCA may develop. Obesity also seems to stimulate the liver to be a "fertile soil" for IHCA development. Hyperleptinemia linked with obesity may contribute to IHCA tumorigenesis by activating leptin/LR-JAK2/STAT3 pathway in tumor while the proinflammation role of leptin with dysfunctional inhibition by SOCS3 in the tumor may contribute to the inflammation status of IHCA. Subsequently, the continuous inflammation of the tumor leads to activation of HPC and HPC mediated regeneration, manifesting as DR. Furthermore, we showed that the peri-PPT areas in IHCA also functions as a stem cell niche in which subclones are present in a constitutive pattern, independent of inflammation and DR.

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NEDERLANDSE SAMENVATTING

Na focale nodulaire hyperplasie (FNH) is het hepatocellulair adenoom (HCA) de tweede meest voorkomende benigne primaire levertumor. HCA komt vooral voor bij vrouwen in de reproductieve leeftijdscategorie na langdurig gebruik van orale anticonceptie. Het inflammatoire type hepatocellulair adenoom (IHCA) is het meest voorkomende HCA subtype en het kan worden geclassificeerd op grond van het genotype. Zestig procent van het IHCA bevat een somatische mutatie van *interleukin-6 signal transducer* (IL6ST), 8-12% toont een mutatie van *signal transducer and activator of transcription-3* (STAT3) en 5% heeft een mutatie van *G-protein alpha stimulatory subunit* (GNAS). Al deze mutaties kunnen leiden tot activatie van STAT3 zonder IL6 binding. Ongeacht het type mutatie toont IHCA een verhoogde expressie van *C-reactive protein* (CRP) en/of *serum amyloid A* (SAA) die immunohistologisch kunnen worden aangetoond en als zodanig de subtypering van IHCA mogelijk maken. Morfologisch wordt IHCA gekenmerkt door de aanwezigheid van ontstekingscellen, sinusoidale dilatatie, solitaire arteriolen, en ductulaire reactie (DR) rond pseudo-portale velden (PPT). Een belangrijk epidemiologisch feit is de associatie tussen IHCA en een hoge body mass index (BMI)/ obesitas.

Dit proefschrift heeft tot doel het verband tussen obesitas en IHCA te onderzoeken en meer inzicht te verkrijgen in de micro-omgeving waarin het IHCA zich ontwikkelt. Wij onderzochten het niet-tumoreuze leverdeel van IHCA; de status van obesitas gerelateerde adipokines en hun receptoren in de tumor en in het niet-tumoreuze deel van de lever; de invloed van leptin op activatie van bloedvatwand-endotheelcellen en leukocyten adhesie in een *in-vitro* studie, en de verschillende eigenschappen en eventuele rol van DR in IHCA.

In **hoofdstuk 2** onderzochten we de histologische en immunohistologische veranderingen van het niet-tumoreuze deel van de lever van 32 gereseceerde IHCA. We hebben afwijkingen gevonden in het niet-tumoreuze deel dat zowel dicht tegen de tumor is gelegen als in het leverweefsel dat verder van de tumor is verwijderd. Geringe tot matige steatose was zeer frequent aanwezig hetgeen overeenstemt met de hoge mediaanwaarde van de BMI (32,55 ± 4.9) van de

studiepopulatie. Gedilateerde sinusoïden en solitaire arteriolen werden regelmatig gezien, zelfs in het lever weefsel dat verder gelegen is van de tumor. Een belangrijke bevinding is de aanwezigheid van kleine CRP-positieve foci in circa 50% van de gevallen waarin kleine IHCA foci werden gezien, vooral voorkomend bij IHCA patiënten met meerdere noduli. Dit suggereert dat het niet-lesionele deel van een IHCA leverafwijkingen bevatten die potentieel de groei van nieuwe adenomen kunnen stimuleren. Deze bevindingen zouden het klinisch follow-up beleid kunnen beïnvloeden, temeer omdat het bekend is dat obesitas de groei van maligne tumoren kan stimuleren.

De studies in **hoofdstuk 3** zijn gebaseerd op de hypothese dat obesitas de tumorigenese in IHCA positief kan stimuleren door secretie van adipokines door het viscerale vetweefsel en door een directe targeting van de lever via de portale circulatie. We hebben in dit hoofdstuk de expressie bestudeerd van leptin receptor (LR) en adiponectin receptor 2 (AdiR2) in 25 IHCA en vonden een verhoogde genexpressie van het LR gen in de tumor ten opzichte van het niet-lesionele lever deel en normale controle lever. Het AdiR2 gen was verlaagd in de tumor. Activatie van de leptin-LR pathway en de downstream signaling effecten ervan werden aangetoond door de aanwezigheid van nucleaire expressie van p-STAT3 en een toename van STAT3 genexpressie in de tumor. LR expressie werd vooral gezien op het sinusoidaal endotheel terwijl nucleaire p-STAT3 expressie niet alleen aanwezig was in de kernen van hepatocyten maar ook in de kernen van de sinusendotheelcellen (SEC). In een *in vitro* studie vonden we dat leptin stimulatie tot op-regulatie van IL-6 genexpressie leidde in zowel humane navelstrengendotheelcellen *(human umbilical vein endothelial cells* (HUVEC)) als in humane leversinusendotheelcellen (*human hepatic sinusoidal endothelial cells* (hHSEC)). Deze data, die voor het eerst een verband toonden tussen obesitas en IHCA, suggereren dat leptin een stimulerende invloed kan uitoefenen op de inflammatie en op de proliferatie status van IHCA.

Op grond van de bevindingen dat het mRNA niveau van LR opgereguleerd was in IHCA en dat LR tot expressie werd gebracht in SEC namen wij aan dat leptin een pro-inflammatoire rol zou kunnen hebben via de binding met LR op SEC en hiermee een bijdrage levert tot het inflammatoire fenotype van IHCA. We

hebben daarom in **hoofdstuk 4** ons onderzoek gericht op de pro-inflammatoire invloeden van de leptin-LR activatie in HUVEC en hHSEC. Beide types endotheelcellen brengen LR tot expressie en STAT3 werd snel geactiveerd na stimulatie met leptin. Tegelijkertijd werd de negatieve *feedback loop* van STAT3 activatie geactiveerd zoals aangetoond door de opregulatie van het mRNA niveau van *suppressor of cytokine signaling 3* (SOCS3). Het mRNA niveau van *intercellular adhesion molecule-1* (ICAM-1) in HUVEC en hHSEC werden in geringe mate opgereguleerd en er was ook een toegenomen ICAM-1 eiwit expressie in HUVEC, maar dit resulteerde niet tot een toename van HL60 leukocyten adhesie. Er was ook geen effect van leptin stimulatie op leukocyten adhesie na TNFα geïnduceerde endotheel activatie.

De micro-omgeving van IHCA in patiënten is waarschijnlijk veel gecompliceerder dan onze experimentele nabootsing in **hoofdstuk 4**. Naast hyperleptinemie hebben patiënten met obesitas/overgewicht bijvoorbeeld ook hypoadiponectinemie en een hogere serumspiegel van TNFα. Hypoadiponectinemie en een lagere genexpressie van AdiR2 in de tumor zullen leiden tot een reductie van de beschermende effecten van adiponectin en verzwakking van de tegenwerking van leptin in IHCA, terwijl serum TNFα mogelijkerwijs de voornaamste en krachtigste effector is van leukocyten adhesie.

Een continue inflammatoire status en vasculaire afwijkingen zoals gedilateerde sinusoïden zouden tot parenchym schade kunnen leiden. In veel leverziekten wordt DR beschouwd als een *hepatic progenitor cell* (HPC) gemedieerde lever regeneratie. IHCA bevat vaak DR terwijl deze slechts zelden in de andere subtypen van HCA wordt gezien. Het onderzoek van **hoofdstuk 5** is gebaseerd op de hypothese dat DR in IHCA een regeneratieve activiteit vertegenwoordigt. Wij hebben DR waargenomen in 58% van IHCA gevallen terwijl 83% een gering tot matige inflammatie bevatten. De ontstekingscellen zijn gelokaliseerd in de PPT die unieke structuren zijn van IHCA, terwijl DR consequent wordt gezien direct gelegen tegen de PPT. Immunohistologische identificatie van HPC werd verricht door toepassing van de HPC markers CK19, *epithelial cell adhesion molecule* (EpCAM) en *sex determining region Y-box 9* (Sox9). Cellen die positief waren met deze markers werden gezien in DR die cholangiocyten

vertegenwoordigen met HPC eigenschappen. Er werd ook een wisselende hoeveelheid hepatocyten gezien die deze markers tot expressie brachten hoewel het aantal ervan veel lager was dan dat van de ductulaire cellen. Een belangrijke bevinding is de sterke associatie tussen inflammatie en DR en tussen inflammatie en de expressie van HPC markers. Wij hebben voorts de clonale status onderzocht van de peri-PPT gebieden waar DR is gelokaliseerd, gebruik makend van de 2-kleuren enzym histochemie techniek van cytochroom-*c*-oxidase/ succinaat dehydrogenase (CCO/SDH), gevolgd door sequentie analyse van het gehele mitochondriale DNA (mtDNA). Met de laatste methode hebben we een identieke mtDNA mutatie aangetoond in cellen binnen een enkel CCO-deficiënt gebied. Deze mutatie is afwezig in de omgevende CCO-positieve hepatocyten. CCO-deficiënte gebieden die ieder een clonale celpopulatie vertegenwoordigen waren aanwezig in 67% van IHCA. Deze CCO-deficiënte gebieden, die cellen bevatten met een verlies van CCO productie werden consequent gevonden in peri-PPT gebieden. Er is echter geen associatie tussen het voorkomen van deze clonale celpopulaties met de aanwezigheid van inflammatie, DR of expressie van HPC markers. Desalniettemin zijn deze clonale populaties afkomstig van stamcellen. De waarneembare CCO enzymdeficiëntie kan namelijk uitsluitend bereikt worden na uitgebreide accumulatie van mtDNA mutaties waarvoor een dusdanig lange cellulaire levensduur noodzakelijk is dat slechts een stamcel dat kan bereiken.

Deze bevindingen toonden aan dat DR in IHCA een HPC gemedieerde regeneratie respons vertegenwoordigt terwijl de clonale expansie een structureel patroon laat zien. Ondanks het feit dat beide fenomenen in dezelfde locatie plaatsvinden, die als de stamcel niche beschouwd kan worden, hebben de 2 processen verschillende pathogenetische achtergronden.

We hebben in dit proefschrift de micro-omgeving van IHCA onderzocht en vonden aanwijzingen dat obesitas zowel de lever waarin IHCA zich ontwikkelt als het IHCA zelf zou kunnen beïnvloeden, waarschijnlijk via de adipokines die zowel de groei als het inflammatoire fenotype stimuleren. De inflammatie kan een HPC-gemedieerde regeneratie respons induceren terwijl de HPC/stamcel niche subclonale populaties genereert onafhankelijk van de inflammatoire status.

Translated by Annette S.H. Gouw

中文摘要

肝细胞腺瘤（HCA）是继肝局灶性结节增生之后的第二大常见的良性肿瘤，有长期使用口服避孕药的可孕女性是 HCA 的高发人群。炎症性的肝腺瘤（IHCA）是 HCA 的主要类型，可以突变基因型不同再分类。60%的 IHCA 包含白细胞介素 6 信号转导因子(IL6ST)的体细胞突变，8～12% 含有信号转导和转录因子- 3（STAT3）的突变，5%则含有兴奋性 G 蛋白 alpha 亚基突变（GNAS），这三种类型的突变都可以无需结合 IL-6 配体而激活下游的 STAT3。无论何种突变类型，IHCA 在免疫组化的诊断中都显示为 C-反应蛋白（CRP）和/或血清淀粉样蛋白 A（SAA）的高表达。形态学上，IHCA 特征为浸润的炎性细胞、肝血窦扩张、异常动脉和伪门管区（PPT）的胆管增生反应（DR）。一个重要的流行病学特征是 IHCA 的发生与具有较高的身体质量指数（BMI）的女性有关联 。

在本论文中，我们的目的是研究肥胖与 IHCA 的发生是否存在病理学上的联系，并且深入探索 IHCA 发生发展的微环境。我们观察了 IHCA 病人非肿瘤的肝脏部位；与肥胖相关的脂肪分泌因子受体在肿瘤与非肿瘤的肝脏中的表达状态；在体外实验中检测了瘦素对内皮细胞与白细胞粘附的影响；以及探讨 DR 在 IHCA 中的作用。

第二章中，我们分析了 32 例 IHCA 病人非肿瘤肝脏部位的病理学与免疫组织化学方面的改变，发现在邻近肿瘤的肝脏区域与远离肿瘤的肝脏区域都有异常的表现。与病人高中位数的 BMI（32.55±4.9）一致，多数的病例中出现轻度到中度的肝脂肪变性。在远离肿瘤的肝脏中也常出现扩张的肝血窦与异常动脉。大约一半的病例中出现 CRP 阳性灶说明了在有多个肿瘤灶 IHCA 病人的非肿瘤的肝脏部位中隐匿着微小腺瘤。这个重要发现预示了非肿瘤灶肝脏部位的微环境已经发生了改变，具有了发展为潜在新腺瘤的倾向。这些发现将会影响随访，特别是肥胖已经被认为是可以促进恶性肿瘤发生的危险因素。肥胖可能也参与到 IHCA 的肿瘤发生，因为内脏周边的脂肪组织可以产生脂肪分泌因子通过门静脉直接作用于肝细胞，基于以上的假设我们设计了第三章的研究。

在这一章中，我们检测了瘦素受体（LR）与脂联素受体 2（AdiR2）在 25 例 IHCA 病人中的表达情况，并发现 LR 基因在肿瘤中比非肿瘤部位和正常的肝脏中表达高，而 AdiR2 基因在肿瘤中则较低。瘦素以及 LR 的通路激活以及其下游的效应表现为肿瘤中磷酸化 STAT3 的表达以及较高的 STAT3 基因的表达。LR 主要表达在肝血窦内皮上(SEC)，而磷酸化的 STAT3 不仅表达在肝细胞核里也表达在肝血窦内皮的细胞核中。在体外实验中，瘦素刺激后的人类脐带静脉内皮细胞（HUVEC）与人类肝脏血窦内皮细胞（hHSEC）都上调了 IL-6 的基因表达。 这些结果预示了瘦素可能参与了 IHCA 的炎症表型以及肿瘤增生，同时正表明了肥胖与 IHCA 的关联。

基于 LR 的基因在 IHCA 肿瘤中表达较高而且主要定位在肝血窦内皮上，我们设想在 IHCA 病人中，瘦素可能会通过与 SEC 上的受体结合而促进炎症发生。因此，第四章主要分析了在 HUVEC 与 hHSEC 中瘦素-LR 激活后的促炎影响。这两种类型的内皮细胞都表

达 LR，并且在瘦素的作用下短时间内 STAT3 迅速的被激活。同时 STAT3 激活的负反馈也被激活，表现为细胞因子信号转导抑制因子 3（SOCS3）基因的上调。这两种内皮的细胞间粘附分子 1 基因水平都有轻微上调，其蛋白水平在 HUVEC 也有相应的上调，但是不足以引起 HL60 白细胞粘附的增加。在与肿瘤坏死因子α（TNFα）共同作用于内皮细胞也没有发现瘦素对白细胞粘附的影响。

在 IHCA 病人体内的微环境很有可能比我们在第四章模拟的复杂很多。例如，这些肥胖或具有超标体重的个体，不仅有高瘦素血症，还共存低脂联素血症以及较高的 TNFα 血清水平。 低脂联素血症及肿瘤中低表达的脂联素受体可能会导致在 IHCA 病人中的保护以及拮抗瘦素的作用减弱，而血清 TNF-α可能是白细胞粘附中起重要作用的因子。

IHCA 中持续炎症的状态以及扩张的血窦等血管异常的表现会造成肝细胞实质的损伤。在许多肝脏疾病中，DR 被认为是肝前体细胞（HPC）调节肝脏再生，而且多数 IHCA 具有 DR，却罕见于 HCA 的其他类型。

因此，我们在第五章中提出假设，DR 在 IHCA 中可能发挥着肝脏再生的功能。我们发现 58%的 IHCA 有 DR 的表现而且 83%的病例有轻/中度炎症。炎症细胞主要聚集在 IHCA 特征之一的 PPT 附近，而 DR 也出现在 PPT 周边。我们采用了 HPC 的特异性抗原 CK19，上皮细胞粘附因子（EpCAM）以及 Y 性别决定域转录因子 9（Sox9）在 IHCA 中以免疫组化的方式标记 HPC。这些抗原阳性的细胞都在 DR 中表明了胆管上皮具有 HPC 的特性，也有少量肝细胞表达这些抗原。重要的是炎症发生与 DR 以及 HPC 表达直接相关联。随后我们用双组织酶学染色细胞色素 c 氧化酶/琥珀酸脱氢酶（CCO/SDH）检测了 PPT 周边 DR 所在区域的克隆状态以及其线粒体 DNA 的测序。通过线粒体 DNA 的测序分析表明在 CCO 缺失的肝细胞比 CCO 阳性的肝细胞存在突变表型。代表着突变细胞群的 CCO 缺失的区域出现在 67%的 IHCA 的 PPT 周边。尽管其出现与炎症发生以及 DR 或 HPC 表达在 IHCA 中没有直接的相关，但是单一 CCO 缺失肝细胞区域预示着由同一干细胞演变而来。因为只有经历过长期的过程而且积累了一定量的线粒体基因突变的干细胞才可以演变成可见的 CCO 缺失的区域。

这些发现表明了 DR 在 IHCA 病人中代表着 HPC 调节的肝脏再生，而克隆的演变是一个持续的过程。尽管这两种现象都发现在干细胞巢相同位置，但是有着不同的病理背景。

综上所述，在关于 IHCA 微环境的论文研究中，我们发现 IHCA 病人非肿瘤病灶肝脏部分的微环境发生了改变，已经具有发展为潜在新的腺瘤的倾向；肥胖通过释放脂肪分泌因子而促使肿瘤增生与炎症发生，可能会影响非肿瘤的肝脏演变同时也影响 IHCA 的肿瘤发生；而炎症的发生则引起 HPC 调节的肝细胞再生；HPC 或干细胞的克隆演变与炎症发生没有存在直接的关联。

校对 韩金珠

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ABOUT THE AUTHOR

Jing Han was born on 10th May, 1983 in Henan, China. After graduation from Xinxiang No.1 high school, she studied Clinical Medicine in Xinxiang Medical University from 2002. In 2007, she gained her Bachelor Degree on Clinical Medicine in Xinxiang. Subsequently, she started the successive postgraduate and doctoral program in the department of Pathology of Fudan University Shanghai Medical College. In 2009, she obtained the certification of Medical Doctor in Shanghai, after which she was involved in a study of tracing early hepatocellular carcinoma using Iodine-131 labeling of membranous hepatocellular carcinoma markers. In the same year, she was recommended to start her PhD study on hepatocellular adenomas in the department of Pathology at the University Medical Center Groningen. Since she came to the Netherlands on 15th Dec. 2010, she worked on the research presented in this thesis entitled “The Microenvironment of the Inflammatory Type Hepatocellular Adenoma”.