The way forward in hepatoblastoma : a study of epidemiology, gene expression patterns, and the development of a tumor model

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The way forward in hepatoblastoma

A study of epidemiology, gene expression patterns, and the development of a tumor model

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Faculteit der Geneeskunde

Contents

Chapter 1

Where do we stand with hepatoblastoma?

A review

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SE Köhler[2]
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Contents

1.1 A short history

Over a century ago, Misick, then at the Pathological-Anatomical Institute of Professor Chiari in Prague, probably published the first case report of a hepatoblastoma (HB) in the English literature[1]. It was titled 'A case of teratoma hepatis' and described a 6-weeks old boy who died of respiratory problems. Post-mortem examination revealed 'a large tumor mass, about the size of a man's fist, which occupied the lower half of the right lobe of the liver'. On dissection, a gray-yellow mass with numerous lobules separated from each other by connective tissue septa was seen. Furthermore, smooth-walled cysts of various size and numerous cartilaginous and bone-like deposits were observed. The branches of the hepatic vein were infiltrated by the tumor. Microscopically, embryonal-appearing hepatocytes, spindle-cell sarcomatous stroma, gland-like spaces, osteoid with osteoblasts and squamous epithelium were identified. Dehner and Manivel therefore concluded that it was not surprising that Misick described this neoplasm as a teratoma with tissue representatives of the three embryonic germ-cell layers[2]. More than 60 years later, Willis introduced the term 'hepatoblastoma' for this type of tumor and proposed to use this term in 'all embryonic tumors containing hepatic epithelial parenchyma'[3]. At that time, the distinction between HB and hepatocellular carcinoma (HCC) in children was often not made. Moreover, the three subtypes that were distinguished by Willis based upon the pure and mixed epithelial and mesenchymal appearances within the tumor: embryonic hepatoma, mixed tumor, and rhabdomyoblastic mixed tumor, only added to the confusion. Even in 1986, when in fact clear morphologic criteria had been defined for HB and HCC largely by the work of Ishak and Glunz in 1967[4], another morphologic variant was introduced by Manivel, namely 'teratoid hepatoblastoma'[5]. This demonstrates once again the confusion that existed and, maybe, still exists in the diagnosis of this relatively rare pediatric liver malignancy. Anyhow, the interest in this childhood tumor has increased tremendously the last 3 decades as is reflected by the increase in the annual number of publications found in Medline from 2 in 1965 to 116 in 2004. In the last decade, several excellent clinical reviews regarding liver tumors in children have appeared, but a review of the biological aspects of HB is lacking[6-10]. In addition to diagnostic characteristics and current treatment modalities for patients with HB, this review discusses the currently known phenotypic features, cytogenetic alterations, and possible pathogenetic role of cytokines, β-catenin and the Wnt signaling pathway of this malignant tumor in more detail. The scope of this thesis is explained in the last paragraph.

1.2 Epidemiology

1.2.1 Incidence

Hepatoblastoma is a malignancy of the liver with a fairly constant annual incidence of 0.5-1.5 diagnoses per 1 million children age younger than 15 years in Western countries[11], although an increase has been reported in the U.S. It comprises 1% of all pediatric malignancies and affects

mostly infants and young children between the ages of 6 months and 3 years, although neonates and adolescents with HB have been reported. After neuroblastoma and nephroblastoma, primary epithelial tumors of the liver are the third most common intraabdominal neoplasms in children[12]. Hepatoblastoma is the most frequent liver tumor in Western countries. In Asia and Africa, hepatocellular carcinoma (HCC) occurs more frequently than HB, probably as a consequence of the greater prevalence of hepatitis B infection on those continents.

1.2.2 Risk factors

To date, no environmental risk factors for HB have been described; however, HB has been associated recently with prematurity or a low birth weight[13,14]. Familial cases have been reported. In this respect, the coincidence of HB with familial adenomatous polyposis (FAP) and Beckwith-Wiedemann syndrome (BWS) is striking and suggests a role in the pathogenesis of HB for chromosomes 5 and 11, respectively[15,16].

1.3 Etiology

1.3.1 Cytogenetic alterations

Cytogenetic analysis of HB has not revealed a consistent pattern of chromosomal anomalies. The most common genetic aberrations are extra copies of chromosomes 1q, 2q, 7q, 8, 17q, and 20[16-22]. However, to date, cytogenetic alterations have not been linked with a causal factor or with prognosis[23]. Of more functional importance, loss of heterozygosity (LOH) of 11p15 has been observed in up to one-third of patients with HB, and LOH of chromosome 1p also in approximately 33% of patients with HB[24,25]. Loss of heterozygosity at 11p15, which is always of maternal origin[25], is nearly pathognomonic for patients with BWS, who have a greater risk of developing HB, Wilms' tumor, and rhabdomyosarcoma[16]. Important imprinted genes on 11p15.5 are *p57KIP²*, *insulin-like growth factor 2* (*IGF-2*) and *H19*. *p57KIP²* and *H19* are tumor suppressor genes, whereas *IGF-2* is a major fetal mitogen. In this context, it is noteworthy that *IGF-2* transcription is affected by β-catenin mutations, which also seem to play a role in the development of HB. *p57KIP²* is up-regulated in HB[26], which argues against its role as a suppressor gene. Loss of imprinting is noted for the maternally imprinted *IGF-2* gene but, in HB, is not associated with increased expression of *IGF-2* or decreased expression of *H19*, as it is in BWS and its other associated tumors[16]. Mutations observed in the tumor suppressor gene *p53* that often are reported in HB appear to play no pathogenetic role in the development of HB, because they are found in a large portion of all tumors[16,27-29]. Furthermore, overexpression of *p53* was not correlated with patient survival[30].

1.3.2 Pathogenesis

The pathogenetic mechanisms responsible for the development of HB remain unclear. HB, as an *embryonal tumor*, is derived from undifferentiated embryonal tissue. Rapidly growing HBs often are very sensitive to cytotoxic drugs and sometimes are very sensitive to radiotherapy[31,32].

1.3.2.1 *Histological clues*

The currently accepted hypothesis is that HB cells are derived from pluripotent hepatic stem cells[33-35]. These stem cells, or oval cells, have retained the ability to differentiate into both hepatocytes and biliary epithelial cells and, accordingly, express markers for both cell types, a feature also found in HB[36]. In addition, extramedullary intratumoral erythropoiesis and thrombopoiesis are found in HB[37], a feature normally present in the fetal liver.

1.3.2.2 *Role of cytokines*

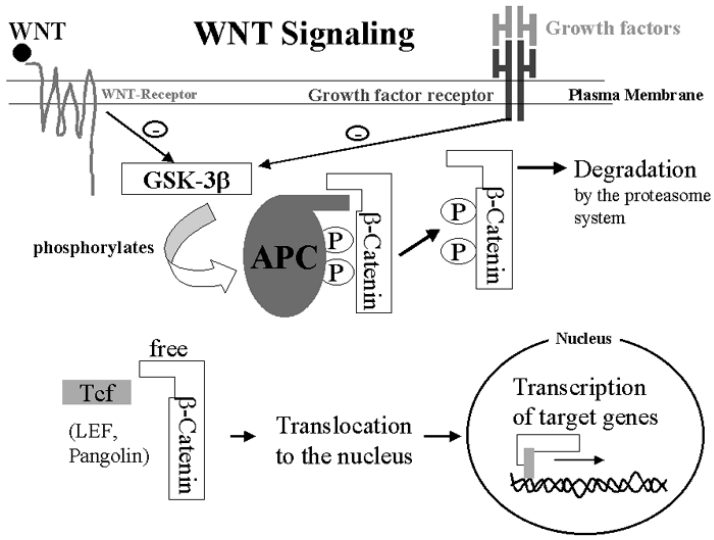
Hepatoblastoma cells secrete interleukin 1β (IL-1β), which, in turn, induces IL-6 production in the surrounding fibroblasts and endothelial cells[38]. Secreted IL-6 may induce the production of the acute phase protein β-2-microglobulin. Both IL-1β and IL-6 can stimulate the secretion of *hepatocyte growth factor* (*HGF*). *Hepatocyte growth factor* is expressed in childhood HB by fibroblasts and endothelial cells and functions as a paracrine growth factor for HB cells that express the *HGF* receptor c-met[39,40]. The rapid growth of recurrent, disseminated tumor and/or metastases that can occur in children with incompletely resected, nonpretreated HB may result from increased *HGF* secretion after they undergo hepatic resection[41].

1.3.2.3 *Pathogenetic pathway*

Epidemiological studies revealed that HB occurs more often in families affected by FAP[42]. Familial adenomatous polyposis is caused by inactivation of the *adenomatous polyposis coli* (*APC*) tumorsuppressor gene that is localized on chromosome 5. The function of this gene is the down-regulation of β-catenin. In greater than 67% of patients with sporadic HB, alterations of the APC gene were observed[43]. However, similar numbers of patients also show activating mutations of the β-catenin gene[44-46]. Together, these data suggest that the Wnt signaling pathway plays a role in the development of HB. APC binds to β-catenin, thereby promoting its NH_2-terminal phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK-3β). This phosphorylation targets β-catenin for degradation by the proteasome system. Signaling through the Wnt signal-transduction pathway (see FIGURE 1) and deletions of the NH_2-terminus of β-catenin inhibit this phosphorylation by inhibiting GSK-β3 and, thus, cause accumulation of unphosphorylated β-catenin in the cytosol. So called *activating mutations* of β-catenin, in which the amino terminal phosphorylation target sequences are mutated or deleted, also cause accumulation of β-catenin in the cytosol. This β-catenin then translocates to the nucleus, where it interacts with the HMG-box transcription factors lymphoid enhancer factor 1, T-cell factor 3 (Tcf3), Tcf4, and pangolin to modulate the transcription of target genes such as *c-Myc*,

cyclin D1, *matrix metalloprotease-7*, *immunoglobulin transcription factor-2*, and *fibronectin* (Figure 1). Whereas the absence or presence of mutations in β-catenin is not of prognostic value, a predominantly nuclear (as opposed to cytosolic) localization of β-catenin shows significant correlation with shorter survival time in these patients[47]. This most likely is because overexpression of the target genes of β-catenin may support HB progression[48]. In this respect, it is intriguing that *HGF* can also induce transient β-catenin translocation to the nucleus in a Wnt independent manner (Figure 1)[49]. This may be one of the ways in which *HGF* exerts its described growth-promoting effect on HB[40].

Figure 1 *The Wnt signaling pathway in relation to β-catenin and hepatoblastoma*



APC: adenomatous polyposis coli; GSK-3β: glycogen synthase kinase 3-β; LEF: lymphoid enhancer factor; P: phosphorylated; Tcf: T-cell factor.

1.4 Diagnosis

1.4.1 Clinical features

Hepatoblastoma is a tumor that typically affects infants and children younger than 3 years. There is a male predominance, and the tumor most likely occurs more frequently among white patients and in the right lobe of the liver[7,50]. HB usually presents as an asymptomatic abdominal mass. Weight loss, anorexia, emesis and abdominal pain indicate advanced disease[9]. Distant metastases, which are found in approximately 20% of patients at diagnosis, occur mostly in the lungs[51], but metastases of the central nervous system and even eye metastases have been described[52,53].

1.4.2 Laboratory findings

Anemia and thrombocytosis are common findings in patients with HB[6]. This finding most likely is related to the ability of HB cells to secrete IL-1β, which, in turn, induces IL-6 production in the surrounding fibroblasts and endothelial cells (see *Role of cytokines*, 1.3.2.2). A recent study including patients with (non-HB) malignancies and a mouse model showed an IL-6 dependent increase in thrombopoietin (TPO) in both patients and mice and a concomitant increase of platelets in the mice[38,54]. This finding appears to explain the increased TPO levels and the commonly found thrombocytosis in patients with HB[6].

A sensitive, but nonspecific marker for the presence of HB is α-fetoprotein (AFP). Approximately 90% of patients with HB have highly elevated serum level of AFP[55], which makes AFP a useful clinical marker for monitoring treatment effectiveness and tumor recurrence. Physiologically, high levels of AFP are expressed in the fetus, with concentrations declining to adult levels in the first 6 months after birth. α-Fetoprotein concentrations can be elevated in patients who have liver diseases associated with liver regeneration, i.e., hepatitis, cirrhosis, hemangioendotheliomas, HCC, germ cell tumors, testicular tumors, and gall bladder carcinomas.

1.4.3 Radiologic findings

Imaging techniques play an important role in the diagnosis, staging, and treatment of patients with HB. Because complete surgical resection is the cornerstone of permanent cure, exact localization and assessment of tumor extent is a prerequisite. Often, the initial diagnosis is made by abdominal ultrasound. Hepatoblastoma presents on abdominal ultrasound as a well defined hyperechoic, solid, usually non-cystic, intrahepatic mass, frequently (60-70%) located in the right lobe of the liver[56]. Characteristically, abdominal computed tomography (CT) scanning reveals a delineated mass with low attenuation compared with the surrounding normal liver parenchyma[57]. Vascular involvement can be assessed with contrast enhancement. Both CT scanning and magnetic resonance imaging also can assess the segmental extension of the tumor and the exact topography of the hepatic vasculature. Angiography can be valuable, although the majority of centers reserve angiography for patients with more complicated disease. Lung metastases are detected with chest X-ray or CT scanning[51]. Bone scanning is not recommended as a routine investigation because bone metastases are rare and bone scans may produce potentially misleading results[58].

1.4.4 Diagnostic Biopsy

A diagnostic biopsy is often omitted if the intention is to treat a tumor that is confined to a single liver lobe with surgery only[59,60]. Nevertheless, a biopsy often is recommended to all patients for accurate diagnosis. First, it may be unethical to give chemotherapy if there is no tissue diagnosis. Second, the elevated physiological expression of AFP may persist after the age of 6 months. Finally, HCC, although it is very rare, has been reported in patients younger than 3 years, and the prognosis for patients with HCC is much worse compared with the prognosis for patients with HB. Thus, we believe that it is advisable for all patients to undergo a biopsy.

The risk associated with a biopsy is low using current techniques, although complications like bleeding and infection do occur in 5-10% of patients[61]. Furthermore, tumor spill after a percutaneous needle biopsy has been described[62].
Histological examination is the only way to ascertain the diagnosis, especially in patients with *nonclassic* tumors. Furthermore, to develop new treatment strategies and to improve outcome, pretreatment phenotypical classification of the primary tumor seems imperative.

1.5 Staging

1.5.1 Clinical staging

Until 1990, all systems for staging primary liver tumors (in children), including HB, were based on the findings *at surgery* or *after surgery*. Then, a staging system that was based exclusively on images obtained *prior to surgery* was developed in the first study of the International Society of Pediatric Oncology Liver Tumor Study Group (SIOPEL-1)[63]. This offered the possibility of including patients who did not undergo surgery, and the information obtained could be used to assess and, if necessary, adjust preoperative therapy. Nevertheless, both preoperative and postoperative staging systems use the same parameters for staging, namely, size, vascular invasion, extension and complexity of the primary tumor, and the absence or presence of metastases.

1.5.1.1 *The postoperative staging systems*

The so-called TNM classification system, originally developed by Pierre Denoix between 1943 and 1952, was adopted by the International Union Against Cancer in 1958[64]. The TNM classification system and stages are summarized in Table 1. In 1983, the Japanese committee on the TNM classification system modified the TNM system, defining T classification solely according to the number of anatomical liver segments involved in the tumor: T1: tumor in one segment; T2: tumor in two segments; T3: tumor in three segments; and T4: tumor in four or more segments[65]. A further simplification of staging liver tumors was implemented by the Children's Cancer Study Group (CCSG) and the Pediatric Oncology Group (POG)[66,67], who described the following four stages: Stage I: complete resection of the tumor; Stage II: microscopic residual tumor; Stage III: macroscopic residual tumor; and Stage IV: distant metastases.

Table 1 *The TNM staging system*[a]

Status/stage	Criteria
Tumor classification	
T1	Solitary, ≤ 2 cm, no vascular invasion, 1 lobe, no extrahepatic disease
T2	Solitary, ≤ 2 cm, vascular invasion, 1 lobe, no extrahepatic disease
T2	Not solitary, ≤ 2 cm, no vascular invasion, 1 lobe, no extrahepatic disease
T2	Solitary, > 2 cm, no vascular invasion, 1 lobe, no extrahepatic disease
T3	Solitary, > 2 cm, vascular invasion, 1 lobe, no extrahepatic disease
T3	Not solitary, ≤ 2 cm, vascular invasion, 1 lobe, no extrahepatic disease
T3	Not solitary, > 2 cm, with or without vascular invasion, 1 lobe, no extrahepatic disease
T4	Not solitary, any size, with or without vascular invasion, > 1 lobe, extrahepatic disease
Stage grouping	
Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage IIIA	T3 N0 M0
Stage IIIB	T1-T3 N1 M0
Stage IVA	T4 any N, M0
Stage IVB	Any T, any N, M1

N0: no regional lymph node metastasis, N1: regional lymph node metastasis, M0: no distant metastasis, M1: distant metastasis.

[a] This system specifies the following tumor characteristics (T classification): multiplicity of tumor nodes (solitary or not solitary), tumor size (≤ 2 cm or > 2 cm), vascular invasion (yes or no), involvement of one or more liver lobes (1 lobe or > 1 lobe), and extrahepatic growth (yes or no). Tumor stage is determined by combining T classification, lymph node involvement (N classification) and distant metastases (M classification).

A retrospective analysis of 72 patients who were treated in the German Pediatric Liver Tumor Study HB89 showed that both the TNM classification system and the CCSG/POG staging system had highly significant predictive value for survival ($P = 0.0001$ and $P = 0.0009$, respectively). In that study, the Japanese TNM staging system had a lower predictive value ($P = 0.0161$), and its modified T classification (i.e., based on the number of liver segments involved) was irrelevant with regard to outcome ($P = 0.1359$) (Table 2)[68].

Table 2 *Disease-free survival according to the respective postoperative staging systems in 72 patients from the German Pediatric Liver-Tumor Study HB89 who were analyzed retrospectively*[a]

	Staging system					
	TNM[b]		CCSG/POG[c]		Japanese TNM[d]	
Stage	%	No.	%	No.	%	No.
Stage I	NA	0	100	21	100	1
Stage II	96	24	50	6	91	23
Stage III	77	34	74	38	71	41
Stage IV	29	14	29	7	29	7
P-value[e]	0.0001	–	0.0009	–	0.0161	–

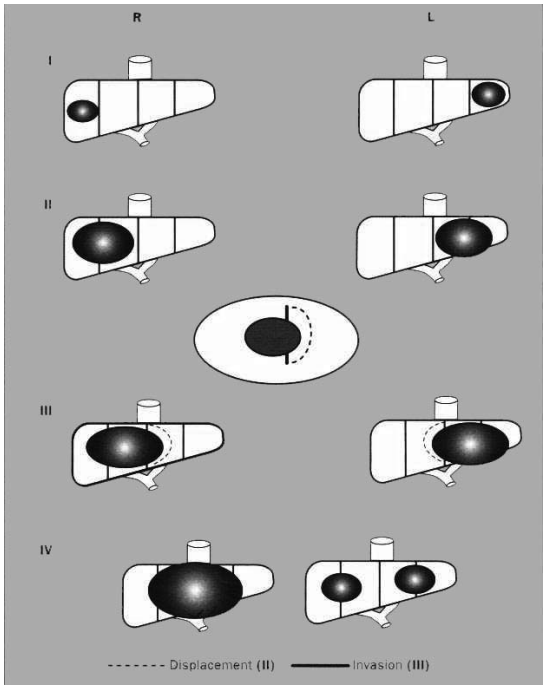
CCSG/POG: Children's Cancer Study Group/ Pediatric Oncology Group; NA: not applicable (did not occur).

[a] See von Schweinitz et al[68]. [b] See Table 1 for definition of stages according to TNM classification system. [c] CCSG/POG: Stage I: complete resection; Stage II: microscopic residual disease; Stage III: macroscopical residual tumor; Stage IV: distant metastases. [d] Japanese TNM: The T classification is defined by the number of segments involved, as follows: T1: tumor in one segment; T2: tumor in two segments; T3: tumor in three segments; T4: tumor in four or more segments. The definitions for N classification and M classification is identical to those used in the conventional TNM system (see Table 1). [e] P values were calculated with the log-rank test and indicate the correlation between disease stage and patient prognosis. P values < 0.05 were considered significant.

1.5.1.2 *The preoperative staging system*

In 1990, SIOPEL adopted a new preoperative staging system, Pretreatment Extent of Disease (PRETEXT). This system is based on the branching pattern of the portal vein, which divides the liver into eight segments, and (non-invasive) imaging techniques. Tumors are classified into one of the four categories by determining the number of affected liver sector(s) (Figure 2). Extrahepatic growth is indicated by adding one or more of the following letters: V: involvement of the hepatic/caval vein; P: involvement of the portal vein; E: the presence of extrahepatic tumor extension; and M: the presence of distant metastases (the VPEM parameters).

Figure 2 *The SIOPEL-1 Pretreatment Extent of Disease grouping system (PRETEXT)*



The system divides the liver into four parts, called sectors. The left lobe of the liver (L) is divided into a lateral sector (Segments 2 and 3) and a medial sector (Segment 4), whereas the right lobe (R) is divided into an anterior (Segments 5 and 8) and a posterior sector (Segments 6 and 7). Tumors are classified into one of the four PRETEXT categories, depending on the number of liver sectors affected by the tumor. PRETEXT I: one sector involved; PRETEXT II: two sectors involved; PRETEXT III: two non-adjoining sectors free or three sectors involved; and PRETEXT IV: all four sectors involved. Extrahepatic growth is indicated by adding one or more of the following characters: V: hepatic/caval vein; P: portal vein; E: extrahepatic extension; and M: distant metastases.

Although the PRETEXT system was developed mainly to assess the efficacy of neoadjuvant chemotherapy and to predict surgical resectability, it also had highly prognostic value for both overall survival and event free survival (Table 3)[69]. Of the VPEM parameters, M (lung metastases) was the only significant parameter that was relevant for survival[51,69]. The predictive, prognostic value for survival of the PRETEXT and TNM classification systems in patients who underwent surgical resection in the SIOPEL-1 study were similar (SIOPEL group, see Chapter IV). This means that the prognostic value of the PRETEXT system is as good as that of the postoperative staging systems but it also allows assessment of the effects of preoperative therapy.

The available systems appear to agree on the major determinants of a patient's prognosis, in that tumor size, tumor extension, and multifocality, all factors that affect resectability directly, are the primary determinants of long-term survival. Distant metastases affect the prognosis negatively. To be able to compare the results of the different study groups, it was decided in 1999 that all international groups would use the SIOPEL PRETEXT system along with their own staging system in their studies[70].

Table 3 *Five-year overall survival and event-free survival according to the PRETEXT staging system in 154 children with HB from the SIOPEL-1 study*[a]

Group[b]	5-year OS (%)	5-year EFS (%)
Group I	100	100
Group II	91	83
Group III	68	56
Group IV	57	46
P-value[c]	0.001	0.0001

OS: Overall survival; EFS: event-free survival.

[a] See Brown et al[69]. [b] Group I: one sector involved; Group II: two sectors involved; Group III: three sectors involved; Group IV: four sectors involved (see also Figure 1). [c] P values were calculated with the log-rank test and indicate the correlation between disease stage and patient prognosis. P values < 0.05 were considered significant

1.5.2 Histologic classification

Currently, there still is no full agreement on the classification of HBs, and refinement of diagnostic criteria for HB is necessary to provide a reproducible classification system[71]. Over 30 years ago, 2 HB subtypes were recognized[4]: the *epithelial* type, which contains predominantly epithelial tissue, and the *mixed epithelial and mesenchymal* type, which also contains tissues of mesenchymal derivation. A classification that was based on the degree of differentiation of HB cells was developed a few years later[72]. Three histologic subtypes were distinguished. The poorly differentiated *embryonal* type was characterized by a tubular or glandular histology and consisted mainly of rosettes of elongated tumor cells as well as varying contributions of fetal cells and anaplastic cells. Hepatoblastoma cells in the highly differentiated *fetal* type resembled normal hepatocytes with rare mitoses and were arranged in two or three cell-thick tumor cords, but a normal lobular architecture was not present. Finally, the *anaplastic* type, also described as the *small cell undifferentiated* type[2], was characterized by small cells with densely stained nuclei and scant cytoplasm. Subsequently, a *macrotrabecular* type of HB characterized by features similar to HCC in adults was added[73]. More recently, an elaborate histological classification with no less than six patterns was developed[74]. Currently, most pathologists have returned to

the original classification of Ishak and Glunz[4] and distinguish only two morphological types of HB[75]. The *epithelial* type contains embryonal cells or fetal cells and often contains a mixture of the two. In areas with well differentiated HB cells, extramedullary hematopoiesis often is quite prominent[4,76]. The *mixed* type contains mesenchymal tissue in addition to the epithelial elements. The simple division of HB into two morphologic types accommodates individual or regional variations within an individual classification and within more elaborate classifications. Irrespective of these extensive and elaborate efforts to develop a histologic classification system for HB, disagreements remain regarding whether a purely fetal histologic phenotype predicts a favorable prognosis[2,60,67,68,77-79] and whether an anaplastic, *small cell* histologic phenotype predicts an unfavorable prognosis[2,78,80]. Nevertheless, the purely fetal HB is the only histologic subtype that currently leads to a change in therapy in the current Children's Oncology Group protocol for HB (Protocol 9645), although it has been only applied to patients with stage I tumors (i.e., completely resected)[71].

1.6 Treatment

The cornerstone of treatment and the only potential cure for patients with HB is complete resection of the tumor. Although this is a long-known truism, dramatic changes in survival were accomplished only in the last 3 decades. Currently, the 5-year survival rate is 75%[81,82]; whereas, 30 years ago, this rate was only 35%[6]. A short overview of the different treatment modalities used that resulted in this dramatic increase in survival is presented below.

1.6.1 Combined chemotherapy and surgery

The key to improved therapeutic results was the discovery that HB is highly sensitive to cytostatic and cytotoxic drugs, such as vincristine, doxorubicin, cyclophosphamide, 5-fluorouracil[83], and cisplatin[84]. Accordingly, it was found that pretreatment with a combination of cisplatin and doxorubicin (the SIOPEL strategy) improved the prognosis of children with HB, and that strategy has remained the main SIOPEL treatment principle. Some study groups opted for primary resection if possible and only began chemotherapy and *second-look* surgery if primary surgery was not possible[59,85]. In contrast, the prospective SIOPEL-1 trial was the first study that had the intention to treat all patients with preoperative neoadjuvant chemotherapy. This strategy was based on the expectation that preoperative chemotherapy lead to shrinkage of the tumor, rendering the tumor more solid, less prone to bleeding, and better delineated from the healthy liver parenchyma, thus making complete resection more likely[9]. In addition, (micro)metastases, if present, would be treated concurrently. The large scale, prospective SIOPEL-1 trial confirmed the positive results of earlier, smaller scale studies.

The objective of ongoing trials is to improve the prognosis of the 25% of patients who die as a result of their disease. Examples of these new trials are the comparison of two chemotherapy regimens (cisplatin/vincristine/fluorouracil vs. cisplatin/continuous infusion doxo-

rubicin) by the Children's Oncology Group[60]; an HB trial in the U.S. (Protocol 9645; cisplatin/vincristine/fluorouracil vs. carboplatin/cisplatin with or without amifostine; available from URL: http://www.cancer.gov/search/clinical_trials/results_clinicaltrials.aspx); the identification of low-risk and high-risk groups, comparing the treatment of patients who have *low-risk* HB using cisplatin monotherapy with the treatment of patients who have *high-risk* HB using intensified cisplatin/carboplatin/doxorubicin (SIOPEL-2 and SIOPEL-3)[51]; and the application of *megatherapy* with carboplatin and etoposide by the German Study Group[32]. It will be interesting to see which of these strategies produce a further improvement in outcome.

Multidrug resistance is a major problem in the therapy of patients with advanced and recurrent HB. This has been linked to an increased expression of the multidrug resistance gene 1 (MDR-1) and the concomitant increase of its gene product *P-glycoprotein* (*P-gp*), after each course of chemotherapy[86]. *P-glycoprotein* is an ATP dependent membrane channel protein that actively transports drugs out of the cell. An inhibitor of *P-gp*, the chemosensitizer PSC833 significantly improved the effects of chemotherapy in an HB cell culture model and in animals xenotransplanted with human HB[87]. Several inhibitors of *P-gp* are undergoing late-stage clinical trials, and promising results have been obtained in patients with hematologic malignancies, although results for patients with solid tumors have been negative[88,89]. Nevertheless, development of *P-gp* inhibitors is ongoing, and clinical trials are being refined, taking into account the complex interactions between the inhibitor and the target cytotoxic drug[90]. A clearer picture of the clinical relevance of *P-gp* inhibitors and their use in the treatment of patients with HB should emerge over the next few years.

1.6.2 Liver transplantation

The first liver transplantation for malignant liver disease was reported in 1968[91]. Although transplantation as treatment for patients neoplastic disease appeared more promising in children than in adults, frequent tumor recurrence was a major problem. In view of the shortage of donor organs, transplantation as treatment for malignant disease generally was not accepted. This attitude has changed recently after results of orthotopic liver transplantation (OLT) demonstrated additional value in the treatment of patients with HB[92-95]. Orthotopic liver transplantation has become a treatment option for children who have a multifocal, bilobar, otherwise unresectable HB without extrahepatic extension of the tumor that responds to chemotherapy[96-98].

1.6.3 Other treatment options

Chemoembolization has been promoted as a pretreatment option to render an unresectable HB resectable[99], even when this tumor is located in the caudate lobe[100]. These results must be interpreted with caution. First, to our knowledge the majority of reports describe only a single patient or very few patients, and no prospective, randomized results are available. Second, post-

operative complications occurred significantly more often in patients who underwent major hepatectomy followed by chemoembolization than in patients who underwent hepatectomy alone[101]. Third, and most important, the recently published study of children who were treated with chemoembolization demonstrated that only two of six children with HB survived with no evidence of disease, and one of those patients underwent OLT[102]. Similarly, radiotherapy and brachytherapy for patients with HB play a minor role as treatment options[31,103].

The potential side effects of preoperative systemic chemotherapy, such as cardiotoxicity, nephrotoxicity, ototoxicity and bone marrow depression, warrant the search for other, less toxic modalities. Additional treatment options for patients who do not respond to chemotherapy or who develop drug resistance, also are necessary. An attractive strategy that already has been tested in *in vivo* HCC models may be the *suicide gene therapeutic approach*[104,105]. The strategy behind this approach is to kill tumor cells selectively by expressing a gene that can convert a membrane-permeable, non-toxic substance (the *prodrug*) into a toxic agent (the *suicide drug*) in tumor cells only, thus avoiding the toxic effects of systemic chemotherapy. This can be achieved by targeting the tumor cells with, for example, a replication-deficient adenovirus carrying suicide genes such as *E. coli* cytosine deaminase or *Herpes simplex* virus thymidine kinase. In addition to the natural predilection of adenoviruses for hepatocytes, the second level of specificity is formed by using a tumor cell specific promoter/enhancer, like the AFP promoter/enhancer, to express the suicide gene in HB only. The developed *in vivo* HB models could be used to test this strategy for HB[106-108].

1.7 Prognosis

The dramatic improvement of the prognosis for children who present with HB in the last 35 years has shifted the attention to improve survival from therapy to the identification and evaluation of risk factors. It has become clear that children with an extrahepatic tumor extension, multifocality, vascular invasion, DNA aneuploidy, and distant metastases have a poor prognosis and, thus, are at *high risk*[51,68,69]. Whether intensified chemotherapeutic regimens or a switch to new chemotherapy drugs will improve the prognosis of patients with high-risk disease remains to be seen. Resectable tumor, a decline in circulating AFP levels during chemotherapy[109], and pure fetal histology[68,77] were correlated positively with prognosis and probably characterize the patients with *low-risk* disease. Trials also are ongoing in which patients with low-risk HB are pretreated using less intensive chemotherapy (SIOPEL-3). These new trials are of particular interest, in that they will evaluate whether the late toxic effects can be decreased in patients with low-risk HB and whether the efficacy of treatment can be increased in patients with high-risk HB.

1.8 Conclusions

Hepatoblastoma is an uncommon liver malignancy that is seen mostly in children younger than 3 years. The dramatic increase in survival that has been observed in the last 3 decades is due mainly to the combination of chemotherapy and surgery. Currently, approximately 75% of children with HB can be cured completely, although large tumor extent, multifocality of the tumor, and metastatic spread are associated with a poor prognosis. Cellular-biologic and molecular-biologic studies are revealing the biologic properties of this embryonal tumor; however, to date, they have not led to the discovery of reliable prognostic factors. The development of new treatment modalities may be the prerequisite for further improvements in the survival of patients with HB.

1.9 Scope of this thesis

Hepatoblastoma is a pediatric liver malignancy which belongs to the so-called embryonic tumors. Treatment modalities have changed in the last 3 decades which in turn has improved survival considerably. This chapter presented a review about the currently known characteristics of this interesting neoplasm. Epidemiological studies, morphological characteristics and the establishment of a tumor model is described in the following chapters.

In *Chapter II* we analyzed the outcome for those patients presenting with a primary liver tumor treated in the last two decades in the Pediatric Surgical Center of Amsterdam. Furthermore, guidelines are presented for the individual physician how to diagnose and manage these relatively rare pediatric malignancies.

In 1990 the International Society of Pediatric Oncology launched the first prospective trial with the aim to pretreat all children with a HB with chemotherapy. They developed a new staging system solely based on imaging techniques called the PRE-Treatment EXTent of disease system (PRETEXT). This system was used to evaluate the tumor response after different courses of chemotherapy and to analyze at what time point surgical resection could be performed. In *Chapter III*, we analyzed the 128 out of the 154 children included in the SIOPEL-1 study who underwent a surgical resection of their HB between 1990 and 1994. In *Chapter IV,* the predictive value of this new staging system in the 128 out of the 154 children included in the SIOPEL-1 study who underwent a surgical resection of their HB between 1990 and 1994 was analyzed. The predictive value of this system was compared with other well-known staging systems as described in paragraph 1.5.1 '*clinical staging*'.

The epidemiological data showed that approximately a quarter of the patients still die as a result of their disease and risk groups could be identified on clinical data. To improve the outcome in the group of children with this infaust prognosis, the tumor must be analyzed in a more detailed manner. In *Chapter V* we investigated the phenotypic characteristics of human HB nodules by testing the expression patterns of different well known architectural markers in liver

tissue and propose new prognostic factors that are based on the architectural and phenotypic properties of the tumor cells within a HB. Finally, in *Chapter VI*, the establishment and characterization of the subcutaneous and first intrahepatic HB tumor model is described, using human HB cell lines. Both models can be used to test alternative and experimental therapeutic strategies like adenoviral suicide gene therapy, novel agents, and new chemotherapeutic protocols.

References

1. Misick OS. A case of teratoma hepatis. *J Pathol Bacteriol* 1898; 5:128-37.
2. Dehner LP, Manivel JC. Hepatoblastoma: an analysis of the relationship between morphologic subtypes and prognosis. *Am J Pediatr Hematol Oncol* 1988; 10(4):301-7.
3. Willis RA. In: Cameron R, Payling Wright G, eds. The pathology of the tumours of children. Springfield: Charles C. Thoma, 1962; 57-61.
4. Ishak KG, Glunz PR. Hepatoblastoma and hepatocarcinoma in infancy and childhood. Report of 47 cases. *Cancer* 1967; 20(3):396-422.
5. Manivel C, Wick MR, Abenoza P, Dehner LP. Teratoid hepatoblastoma. The nosologic dilemma of solid embryonic neoplasms of childhood. *Cancer* 1986; 57(11):2168-74.
6. Exelby PR, Filler RM, Grosfeld JL. Liver tumors in children in the particular reference to hepatoblastoma and hepatocellular carcinoma: American Academy of Pediatrics Surgical Section Survey--1974. *J Pediatr Surg* 1975; 10(3):329-37.
7. Vos A. Primary liver tumours in children. *Eur J Surg Oncol* 1995; 21(1):101-5.
8. Raney B. Hepatoblastoma in children: a review. *J Pediatr Hematol Oncol* 1997; 19(5):418-22.
9. Perilongo G, Shafford EA. Liver tumours. *Eur J Cancer* 1999; 35(6):953-8; discussion 958-9.
10. Herzog CE, Andrassy RJ, Eftekhari F. Childhood cancers: hepatoblastoma. *Oncologist* 2000; 5:445-53.
11. Mann JR, Kasthuri N, Raafat F, Pincott JR, Parkes SE, Muir KR, et al. Malignant hepatic tumours in children: incidence, clinical features and aetiology. *Paediatr Perinat Epidemiol* 1990; 4(3):276-89.
12. Clatworthy HW, Schiller M, Grosfeld JL. Primary liver tumors in infancy and childhood. 41 cases variously treated. *Arch Surg* 1974; 109(2):143-7.
13. Ikeda H, Matsuyama S, Tanimura M. Association between hepatoblastoma and very low birth weight: a trend or a chance? [see comments]. *J Pediatr* 1997; 130(4):557-60.
14. Feusner J, Plaschkes J. Hepatoblastoma and low birth weight: a trend or chance observation? *Med Pediatr Oncol* 2002; 39(5):508-9.
15. Haas OA, Zoubek A, Grumayer ER, Gadner H. Constitutional interstitial deletion of 11p11 and pericentric inversion of chromosome 9 in a patient with Wiedemann-Beckwith syndrome and hepatoblastoma. *Cancer Genet Cytogenet* 1986; 23(2):95-104.
16. Steenman M, Westerveld A, Mannens M. Genetics of Beckwith-Wiedemann syndrome-associated tumors: common genetic pathways. *Genes Chromosomes Cancer* 2000; 28(1):1-13.
17. Swarts S, Wisecarver J, Bridge JA. Significance of extra copies of chromosome 20 and the long arm of chromosome 2 in hepatoblastoma. *Cancer Genet Cytogenet* 1996; 91(1):65-7.
18. Schneider NR, Cooley LD, Finegold MJ, Douglass EC, Tomlinson GE. The first recurring chromosome translocation in hepatoblastoma: der(4)t(1;4)(q12;q34). *Genes Chromosomes Cancer* 1997; 19(4):291-4.
19. Ma SK, Cheung AN, Choy C, Chan GC, Ha SY, Ching LM, et al. Cytogenetic characterization of childhood hepatoblastoma. *Cancer Genet Cytogenet* 2000; 119(1):32-6.
20. Parada LA, Limon J, Iliszko M, Czauderna P, Gisselsson D, Hoglund M, et al. Cytogenetics of hepatoblastoma: further characterization of 1q rearrangements by fluorescence in situ hybridization: an international collaborative study. *Med Pediatr Oncol* 2000; 34(3):165-70.
21. Weber RG, Pietsch T, von Schweinitz D, Lichter P. Characterization of genomic alterations in hepatoblastomas. A role for gains on chromosomes 8q and 20 as predictors of poor outcome. *Am J Pathol* 2000; 157(2):

571-8.
22. Ali W, Savasan S, Rabah R, Mohamed AN. Cytogenetic findings in two new cases of hepatoblastoma. *Cancer Genetics and Cytogenetics* 2002; 133:179-82.
23. von Schweinitz D, Kraus JA, Albrecht S, Koch A, Fuchs J, Pietsch T. Prognostic impact of molecular genetic alterations in hepatoblastoma. *Med Pediatr Oncol* 2002; 38:104-8.
24. Kraus JA, Albrecht S, Wiestler OD, von Schweinitz D, Pietsch T. Loss of heterozygosity on chromosome 1 in human hepatoblastoma. *Int J Cancer* 1996; 67(4):467-71.
25. Albrecht S, von Schweinitz D, Waha A, Kraus JA, von Deimling A, Pietsch T. Loss of maternal alleles on chromosome arm 11p in hepatoblastoma. *Cancer Res* 1994; 54(19):5041-4.
26. Hartmann W, Waha A, Koch A, Goodyer CG, Albrecht S, von Schweinitz D, et al. p57(KIP2) Is Not Mutated in Hepatoblastoma but Shows Increased Transcriptional Activity in a Comparative Analysis of the Three Imprinted Genes p57(KIP2), IGF2, and H19. *Am J Pathol* 2000; 157(4):1393-1403.
27. Debuire B, Paterlini P, Pontisso P, Basso G, May E. Analysis of the p53 gene in European hepatocellular carcinomas and hepatoblastomas. *Oncogene* 1993; 8(8):2303-6.
28. Chen TC, Hsieh LL, Kuo TT. Absence of p53 gene mutation and infrequent overexpression of p53 protein in hepatoblastoma. *J Pathol* 1995; 176(3):243-7.
29. Kusafuka T, Fukuzawa M, Oue T, Komoto Y, Yoneda A, Okada A. Mutation analysis of p53 gene in childhood malignant solid tumors. *J Pediatr Surg* 1997; 32(8):1175-80.
30. Zerbini MC, Sredni ST, Grier H, Cristofani LM, Latorre MR, Hollister KA, et al. Primary malignant epithelial tumors of the liver in children: a study of DNA content and oncogene expression. *Pediatr Dev Pathol* 1998; 1(4):270-80.
31. Habrand JL, Nehme D, Kalifa C, Gauthier F, Gruner M, Sarrazin D, et al. Is there a place for radiation therapy in the management of hepatoblastomas and hepatocellular carcinomas in children? [see comments]. *Int J Radiat Oncol Biol Phys* 1992; 23(3):525-31.
32. von Schweinitz D. Identification of risk groups in hepatoblastoma--another step in optimising therapy [editorial]. *Eur J Cancer* 2000; 36(11):1343-6.
33. Ruck P, Xiao JC, Pietsch T, Von Schweinitz D, Kaiserling E. Hepatic stem-like cells in hepatoblastoma: expression of cytokeratin 7, albumin and oval cell associated antigens detected by OV-1 and OV-6. *Histopathology* 1997; 31(4):324-9.
34. Vassey CJ, De La Hall PM. Hepatic stem cells: a review. *Pathology* 2001; 33:130-41.
35. Ruck P, Xiao JC. Stem-like cells in hepatoblastoma. *Med Pediatr Oncol* 2002; 39(5):504-7.
36. Zimmermann A. Hepatoblastoma with cholangioblastic features ('cholangioblastic hepatoblastoma') and other liver tumors with bimodal differentiation in young patients. *Med Pediatr Oncol* 2002; 39(5):487-91.
37. von Schweinitz D, Schmidt D, Fuchs J, Welte K, Pietsch T. Extramedullary hematopoiesis and intratumoral production of cytokines in childhood hepatoblastoma. *Pediatr Res* 1995; 38(4):555-63.
38. von Schweinitz D, Hadam MR, Welte K, Mildenberger H, Pietsch T. Production of interleukin-1 beta and interleukin-6 in hepatoblastoma. *Int J Cancer* 1993; 53(5):728-34.
39. Miyazaki M, Gohda E, Tsuboi S, Tsubouchi H, Daikuhara Y, Namba M, et al. Human hepatocyte growth factor stimulates the growth of HUH-6 clone 5 human hepatoblastoma cells. *Cell Biol Int Rep* 1992; 16(2):145-54.
40. von Schweinitz D, Faundez A, Teichmann B, Birnbaum T, Koch A, Hecker H, et al. Hepatocyte growth-factor-scatter factor can stimulate post-operative tumor-cell proliferation in childhood hepatoblastoma. *Int J Cancer* 2000; 85(2):151-9.
41. von Schweinitz D, Fuchs J, Gluer S, Pietsch T. The occurrence of liver growth factor in hepatoblastoma. *Eur J Pediatr Surg* 1998; 8(3):133-6.
42. Hughes LJ, Michels VV. Risk of hepatoblastoma in familial adenomatous polyposis. *Am J Med Genet* 1992; 43(6):1023-5.
43. Oda H, Imai Y, Nakatsuru Y, Hata J, Ishikawa T. Somatic mutations of the APC gene in sporadic hepatoblastomas. *Cancer Res* 1996; 56(14):3320-3.
44. Koch A, Denkhaus D, Albrecht S, Leuschner I, von Schweinitz D, Pietsch T. Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the beta-catenin gene. *Cancer Res* 1999; 59(2):269-73.

45. Anna CH, Sills RC, Foley JF, Stockton PS, Ton TV, Devereux TR. Beta-catenin mutations and protein accumulation in all hepatoblastomas examined from B6C3F1 mice treated with anthraquinone or oxazepam. *Cancer Res* 2000; 60(11):2864-8.
46. Udatsu Y, Kusafuka T, Kuroda S, Miao J, Okada A. High frequency of beta-catenin mutations in hepatoblastoma. *Pediatr Surg Int* 2001; 17:508-12.
47. Park WS, Oh RR, Park JY, Kim PJ, Shin MS, Lee JH, et al. Nuclear localization of beta-catenin is an important prognostic factor in hepatoblastoma. *J Pathol* 2001; 193:483-90.
48. Takayasu H, Horie H, Hiyama E, Matsunaga T, Hayashi Y, Watanabe Y, et al. Frequent deletions and mutations of the beta-catenin gene are associated with overexpression of cyclin D1 and fibronectin and poorly differentiated histology in childhood hepatoblastoma. *Clin Cancer Res* 2001; 7:901-8.
49. Monga SP, Mars WM, Pediaditakis P, Bell A, Mule K, Bowen WC, et al. Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. *Cancer Res* 2002; 62(7):2064-71.
50. Hartley AL, Birch JM, Kelsey AM, Jones PH, Harris M, Blair V. Epidemiological and familial aspects of hepatoblastoma. *Med Pediatr Oncol* 1990; 18(2):103-9.
51. Perilongo G, Brown J, Shafford E, Brock P, De Camargo B, Keeling JW, et al. Hepatoblastoma presenting with lung metastases. *Cancer* 2000; 89(8):1845-53.
52. Miyagi J, Kobayashi S, Kojo N, Watanabe M, Kuramoto S, Hirose F. [Brain metastasis of hepatoblastoma--a case report and review of literature]. *No Shinkei Geka* 1984; 12(6):753-8.
53. Endo EG, Walton DS, Albert DM. Neonatal hepatoblastoma metastatic to the choroid and iris. *Arch Ophthalmol* 1996; 114(6):757-61.
54. Kaser A, Brandacher G, Steurer W, Kaser S, Offner FA, Zoller H, et al. Interleukin-6 stimulates thrombopoiesis through thrombopoietin: role in inflammatory thrombocytosis. *Blood* 2001; 98(9):2720-5.
55. Lack EE, Neave C, Vawter GF. Hepatoblastoma. A clinical and pathologic study of 54 cases. *Am J Surg Pathol* 1982; 6(8):693-705.
56. de Campo M, de Campo JF. Ultrasound of primary hepatic tumours in childhood. *Pediatr Radiol* 1988; 19(1):19-24.
57. Siegel MJ, Herman TE. Periportal low attenuation at CT in childhood. *Radiology* 1992; 183(3):685-8.
58. Archer D, Babyn P, Gilday D, Greenberg MA. Potentially misleading bone scan findings in patients with hepatoblastoma. *Clin Nucl Med* 1993; 18(12):1026-31.
59. von Schweinitz D, Burger D, Mildenberger H. Is laparatomy the first step in treatment of childhood liver tumors?--The experience from the German Cooperative Pediatric Liver Tumor Study HB-89. *Eur J Pediatr Surg* 1994; 4(2):82-6.
60. Ortega JA, Douglass EC, Feusner JH, Reynolds M, Quinn JJ, Finegold MJ, et al. Randomized comparison of cisplatin/vincristine/fluorouracil and cisplatin/continuous infusion doxorubicin for treatment of pediatric hepatoblastoma: A report from the Children's Cancer Group and the Pediatric Oncology Group. *J Clin Oncol* 2000; 18(14):2665-75.
61. Bravo AA, Sheth SG, Chopra S. Review Articles: Liver Biopsy. *New Engl J Med* 2001; 344(7):495-500.
62. Takvorian P, Floret D, Valette PJ, Bouffet E, Pradon M. [Hepatic rupture after puncture biopsy: value of embolization. Apropos of a case with hepatoblastoma]. *Pediatrie* 1988; 43(6):531-3.
63. MacKinlay EL, Pritchard J. A common language for childhood liver tumours. *Pediatr Surg Int* 1992; 7:325-26.
64. Sobin LH, Wittekind C, editors. TNM Classification of malignant tumors, 5th edition. New York: John Wiley & Sons, 1997.
65. Morita K, Okabe I, Uchino J, Watanabe I, Iwabuchi M, Matsuyama S, et al. The proposed Japanese TNM classification of primary liver carcinoma in infants and children. *Jpn J Clin Oncol* 1983; 13(2):361-9.
66. Ortega JA, Krailo MD, Haas JE, King DR, Ablin AR, Quinn JJ, et al. Effective treatment of unresectable or metastatic hepatoblastoma with cisplatin and continuous infusion doxorubicin chemotherapy: a report from the Childrens Cancer Study Group. *J Clin Oncol* 1991; 9(12):2167-76.
67. Douglass EC, Reynolds M, Finegold M, Cantor AB, Glicksman A. Cisplatin, vincristine, and fluorouracil therapy for hepatoblastoma: a Pediatric Oncology Group study. *J Clin Oncol* 1993; 11(1):96-9.
68. von Schweinitz D, Hecker H, Schmidt-von-Arndt G, Harms D. Prognostic factors and staging systems in childhood hepatoblastoma. *Int J Cancer* 1997; 74(6):593-9.

69. Brown J, Perilongo G, Shafford E, Keeling J, Pritchard J, Brock P, et al. Pretreatment prognostic factors for children with hepatoblastoma-- results from the International Society of Paediatric Oncology (SIOP) study SIOPEL 1. *Eur J Cancer* 2000; 36(11):1418-25.
70. Plaschkes J. Proceedings of an international research workshop on pediatric liver tumours, 18-20 March, 1999, Berne, Switzerland. Into the year 2000. *Med Pediatr Oncol* 2001; 36(3):380-2.
71. Rowland JM. Hepatoblastoma: assessment of criteria for histologic classification. *Med Pediatr Oncol* 2002; 39(5):478-83.
72. Kasai M, Watanabe I. Histologic classification of liver-cell carcinoma in infancy and childhood and its clinical evaluation. A study of 70 cases collected in Japan. *Cancer* 1970; 25(3):551-63.
73. Gonzalez-Crussi F, Upton MP, Maurer HS. Hepatoblastoma. Attempt at characterization of histologic subtypes. *Am J Surg Pathol* 1982; 6(7):599-612.
74. Stocker JT. Hepatoblastoma. *Semin Diagn Pathol* 1994; 11(2):136-43.
75. Fasano M, Theise ND, Nalesnik M, Goswami S, Garcia de Davila MT, Finegold MJ, et al. Immunohistochemical evaluation of hepatoblastomas with use of the hepatocyte-specific marker, hepatocyte paraffin 1, and the polyclonal anti-carcinoembryonic antigen. *Mod Pathol* 1998; 11(10):934-8.
76. Fuchs J, Schmidt D, Pietsch T, Miller K, von Schweinitz D. Successful transplantation of human hepatoblastoma into immunodeficient mice. *J Pediatr Surg* 1996; 31(9):1241-6.
77. Haas JE, Muczynski KA, Krailo M, Ablin A, Land V, Vietti TJ, et al. Histopathology and prognosis in childhood hepatoblastoma and hepatocarcinoma. *Cancer* 1989; 64(5):1082-95.
78. Conran RM, Hitchcock CL, Waclawiw MA, Stocker JT, Ishak KG. Hepatoblastoma: the prognostic significance of histologic type. *Pediatr Pathol* 1992; 12(2):167-83.
79. von Schweinitz D, Wischmeyer P, Leuschner I, Schmidt D, Wittekind C, Harms D, et al. Clinico-pathological criteria with prognostic relevance in hepatoblastoma. *Eur J Cancer* 1994; 8(8):1052-8.
80. Haas JE, Feusner JH, Finegold MJ. Small cell undifferentiated histology in hepatoblastoma may be unfavorable. *Cancer* 2001; 92(12):3130-34.
81. Pritchard J, Brown J, Shafford E, Perilongo G, Brock P, Dicks-Mireaux C, et al. Cisplatin, doxorubicin and delayed surgery for childhood hepatoblastoma a successful approach. Results of the first prospective study of the international society of pediatric oncology (SIOP) - SIOPEL 1. *J Clin Oncol* 2000; 18(22):3819-28.
82. Schnater JM, Aronson DC, Plaschkes J, Perilongo G, Brown J, Otte J-B, et al. Surgical view of the treatment of patients with hepatoblastoma. Results from the first prospective trial of the International Society of Pediatric Oncology Liver Tumor Study Group (SIOPEL-1). *Cancer* 2002; 94(4):1111-20.
83. Evans AE, Land VJ, Newton WA, Randolph JG, Sather HN, Tefft M. Combination chemotherapy (vincristine, adriamycin, cyclophosphamide, and 5-fluorouracil) in the treatment of children with malignant hepatoma. *Cancer* 1982; 50(5):821-6.
84. Douglass EC, Green AA, Wrenn E, Champion J, Shipp M, Pratt CB. Effective cisplatin (DDP) based chemotherapy in the treatment of hepatoblastoma. *Med Pediatr Oncol* 1985; 13(4):187-90.
85. Reynolds M. Conversion of unresectable to resectable hepatoblastoma and long-term follow-up study. *World J Surg* 1995; 19(6):814-6.
86. Warmann S, Hunger M, Teichmann B, Flemming P, Gratz KF, Fuchs J. The role of the MDR1 gene in the development of multidrug resistance in human hepatoblastoma: clinical course and in vivo model. *Cancer* 2002; 95(8):1795-801.
87. Warmann S, Gohring G, Teichmann B, Geerlings H, Fuchs J. MDR1 modulators improve the chemotherapy response of human hepatoblastoma to doxorubicin in vitro. *J Pediatr Surg* 2002; 37(11):1579-84.
88. Advani R, Visani G, Milligan D, Saba H, Tallman M, Rowe JM, et al. Treatment of poor prognosis AML patients using PSC833 (valspodar) plus mitoxantrone, etoposide, and cytarabine (PSC-MEC). *Adv Exp Med Biol* 1999; 457:47-56.
89. Kaye SB. Multidrug resistance: clinical relevance in solid tumours and strategies for circumvention. *Curr Opin Oncol* 1998; 10 Suppl 1:S15-9.
90. Stein WD. Reversers of the multidrug resistance transporter P-glycoprotein. *Curr Opin Investig Drugs* 2002; 3(5):812-7.

91. Starzl TE, Groth CG, Brettschneider L, Moon JB, Fulginiti VA, Cotton EK, et al. Extended survival in 3 cases of orthotopic homotransplantation of the human liver. *Surgery* 1968; 63(4):549-63.
92. Koneru B, Flye MW, Busuttil RW, Shaw BW, Lorber MI, Emond JC, et al. Liver transplantation for hepatoblastoma. The American experience. *Ann Surg* 1991; 213(2):118-21.
93. Achilleos OA, Buist LJ, Kelly DA, Raafat F, McMaster P, Mayer AD, et al. Unresectable hepatic tumors in childhood and the role of liver transplantation. *J Pediatr Surg* 1996; 31(11):1563-7.
94. Otte JB, Aronson D, Vraux H, de Ville de Goyet J, Reding R, Ninane J, et al. Preoperative chemotherapy, major liver resection, and transplantation for primary malignancies in children. *Transplant Proc* 1996; 28(4):2393-4.
95. Al-Qabandi W, Jenkinson HC, Buckels JA, Mayer AD, McKiernan P, Morland B, et al. Orthotopic liver transplantation for unresectable hepatoblastoma: a single center's experience. *J Pediatr Surg* 1999; 34(8):1261-4.
96. Pichlmayr R, Weimann A, Tusch G, Schlitt HJ. Indications and Role of Liver Transplantation for Malignant Tumors. *Oncologist* 1997; 2(3):164-170.
97. Reyes JD, Carr B, Dvorchik I, Kocoshis S, Jaffe R, Gerber D, et al. Liver transplantation and chemotherapy for hepatoblastoma and hepatocellular cancer in childhood and adolescence. *J Pediatr* 2000; 136(6):795-804.
98. Otte JB, Pritchard J, Aronson DC, Brown J, Czauderna P, Maibach R, et al. Liver transplantation for hepatoblastoma: Results from the International Society of Pediatric Oncology (SIOP) study SIOPEL-1 and review of the world experience. *Pediatr Blood Cancer 2004*; 42(1):74-83.
99. Han YM, Park HH, Lee JM, Kim JC, Hwang PH, Lee DK, et al. Effectiveness of preoperative transarterial chemoembolization in presumed inoperable hepatoblastoma. *J Vasc Interv Radiol* 1999; 10(9):1275-80.
100. Takayama T, Makuuchi M, Takayasu K, LeThai B, Ohyama H, Yamazaki S, et al. Resection after intraarterial chemotherapy of a hepatoblastoma originating in the caudate lobe. *Surgery* 1990; 107(2):231-5.
101. Elias D, Lasser P, Rougier P, Ducreux M, Bognel C, Roche A. Frequency, technical aspects, results, and indications of major hepatectomy after prolonged intra-arterial hepatic chemotherapy for initially unresectable hepatic tumors. *J Am Coll Surg* 1995; 180(2):213-9.
102. Malogolowkin MH, Stanley P, Steele DA, Ortega JA. Feasibility and toxicity of chemoembolization for children with liver tumors. *J Clin Oncol* 2000; 18(6):1279-84.
103. Healey EA, Shamberger RC, Grier HE, Loeffler JS, Tarbell NJ. A 10-year experience of pediatric brachytherapy. *Int J Radiat Oncol Biol Phys* 1995; 32(2):451-5.
104. Kanai F, Lan KH, Shiratori Y, Tanaka T, Ohashi M, Okudaira T, et al. In vivo gene therapy for alpha-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. *Cancer Res* 1997; 57(3):461-5.
105. Cao G, Kuriyama S, Gao J, Nakatani T, Chen Q, Yoshiji H, et al. Gene therapy for hepatocellular carcinoma based on tumour-selective suicide gene expression using the alpha-fetoprotein (AFP) enhancer and a housekeeping gene promoter. *Eur J Cancer* 2001; 37(1):140-147.
106. Pietsch T, Fonatsch C, Albrecht S, Maschek H, Wolf HK, von Schweinitz D. Characterization of the continuous cell line HepT1 derived from a human hepatoblastoma. *Lab Invest* 1996; 74(4):809-18.
107. Fuchs J, Wenderoth M, von Schweinitz D, Haindl J, Leuschner I. Comparative activity of cisplatin, ifosfamide, doxorubicin, carboplatin, and etoposide in heterotransplanted hepatoblastoma. *Cancer* 1998; 83(11):2400-7.
108. Schnater JM, Köhler SE, Bruder E, Bertschin S, Woodtli T, Pietsch T, et al. Ein Tiermodell für das humane Hepatoblastom. In: Siewert JR, ed. Chirurgisches Forum 2002 für experimentelle und klinische Forschung., vol. 31, ed. 1. Berlin: Springer-Verlag, 2002; 543-7.
109. Van Tornout JM, Buckley JD, Quinn JJ, Feusner JH, Krailo MD, King DR, et al. Timing and magnitude of decline in alpha-fetoprotein levels in treated children with unresectable or metastatic hepatoblastoma are predictors of outcome: a report from the Children's Cancer Group. *J Clin Oncol* 1997; 15(3):1190-7.

'A review of the literature is a good mirror to evaluate your own results;
the surgical results of the treatment of liver tumors
in Amsterdam evaluated?'

Chapter 2

Preoperative diagnostic biopsy and surgery in pediatric liver tumors

The Amsterdam experience

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Abstract

Background: To report 24 years of pre-treatment biopsy and surgical experience in primary liver tumors in children.

Methods: Between 1979 and 2003, 53 children presented with a primary liver tumor of whom 48 who underwent surgical resection were evaluated (2 died, 2 were unresectable, and 1 was transplanted). Biopsy data, per- and postoperative complications, mortality, and survival were retrospectively reviewed.

Results: Benign tumors were diagnosed in 8 patients. Surgical resection for a malignant tumor was performed in 40 patients (26 hepatoblastomas (HB), 8 hepatocellular carcinomas (HCC) (4 had fibrolamellar HCC), 3 rhabdomyosarcomas, 1 neuroblastoma, 1 non-hodgkin lymphoma and 1 teratoma). Primary resection was performed in 1 HB, and 4 HCCs. The cumulative survival without evidence of disease was 73% for HB (median 7 yrs) and 88% for HCC (median 3.5 yrs).

Conclusion: The treatment results are comparable with those of larger international series except for HCC. The existing diagnostic pitfalls in differentiating between the various liver malignancies justify the use of a diagnostic biopsy.

Introduction

Primary liver tumors in children are rare. Hepatoblastoma (HB) is the most common with an annual incidence of approximately 1 case per million children <15 years of age in Western countries and accounts for 75% of the primary liver tumors[1,2]. The other 25% comprises other malignant tumors like hepatocellular carcinoma (HCC) and rhabdomyosarcoma (RMS), or benign tumors like hemangiomas, hamartomas or hemangioendotheliomas[3-5]. Surgical resection is unanimously established as the cornerstone of treatment in primary liver tumors, especially if these tumors are malignant. In the last decennia, survival of patients with a hepatoblastoma has improved dramatically due to the introduction of cisplatin in the pre- and postoperative chemotherapy regimens and the improved techniques of liver surgery[6,7], but the prognosis of HCC and RMS remains poor. Against the current 5 years overall survival of HB of about 75%[8-10], stands the survival rate of HCC and RMS of around 20-30%[5,11-13]. Multicenter trials like the studies performed by the Children's Cancer Study Group, the Pediatric Oncology Group, the German co-operative pediatric liver tumor study HB-89, and the SIOPEL-I study of the International Society of Pediatric Oncology (SIOP), have led to this success for HB[8-10,14-16]. It will be interesting to see if the ongoing trials (summarized in *Cancer* 2003;98:668-78) can improve the prognosis for HB further[17]. The distinction between the different liver malignancies has thus prognostic and therapeutic consequences, and should be determined precisely. In this report we retrospectively analyzed our 24 years of surgical experience of treating children with a primary liver tumor and the role of a pre-treatment biopsy. Furthermore, clinical recommendations are presented.

Patients and methods

Between January 1979 and January 2003, 53 children ≤16 years were diagnosed and treated for a primary liver tumor. Patient charts and operation reports were used to collect data regarding demographics, laboratory findings, tumor characteristics (including biopsy), pre- and postoperative staging, operation and follow-up. Two children with a HB died before surgery. The tumors of 3 children (2 with a HB and 1 with a RMS) were unresectable, 1 patient with a HB was transplanted. So 48 children who underwent surgical resection could be evaluated. A subdivision was made into patients with a benign and a malignant tumor. Biopsy data, per- and postoperative complications, mortality, and overall survival of the patients who underwent surgical therapy were analyzed.

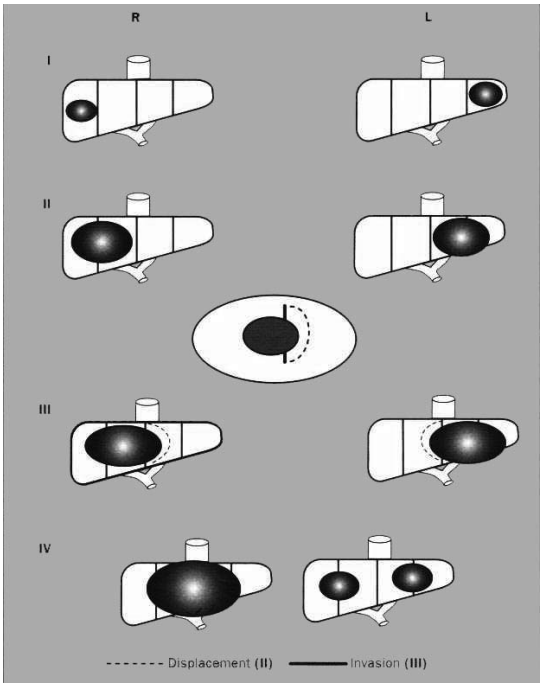
Biopsy technique: Biopsies were either taken as multiple true cut biopsies (n = 22) or via an open technique (n = 10). A standard 14G true cut needle was used. Only on indication, US guidance was performed. If possible, the needle tract was chosen through normal parenchymal tissue, but only if the tract remained within the segment to be removed. In open biopsies, a small wedge biopsy was performed in the periphery of the liver. Hemostasis was secured by both V shaped

sutures, and electro-coagulation at the biopsy site. At the time of tumour resection, biopsy sites were always excised but never contained needle tract metastasis at pathology.

Children with a HB and HCC were staged according to the system proposed by the liver tumor study group of the SIOP (PRETEXT system), in which the number of unaffected liver sector(s) determines the PRETEXT category (Figure 1)[9,18]. Staging was performed retrospectively if not described in the patients charts (applicable to the earlier cases). The PRETEXT is a system based exclusively on imaging techniques, and can therefore be used pre-operatively.

A standard statistical package (SPSS) was used to store and analyze all data. Statistical analysis was only performed for children with HB, the number of children with another type of tumor was too small. The Kaplan-Meier method was used to generate overall survival curves[19], and comparison between the different variables and survival was assessed with the log-rank test[20]. The level of significance was taken at $P < 0.05$.

Figure 1 *The SIOPEL-I staging system*



The PRETreatment EXTent of disease staging system (PRETEXT). Extrahepatic extension is indicated by adding one or more letters as follows: involvement of the hepatic/caval vein (V), portal vein (P), extrahepatic tumor extension (E), and presence of distant metastases (M).

Results

Benign tumors (n = 8)

A benign tumor occurred in 8 children (4 males, median age 7 months, range 0-76 months). A mesenchymal hamartoma was seen in 3 patients, a cyst in 2, a hemangioendothelioma in 2, and a hemangioma in 1. The diagnosis was made solely with laboratory findings and imaging techniques in 5 patients, whereas a pre-operative true cut histological biopsy was added to distinguish between a benign or a malignant tumor in 3 patients. In 7 patients primary resection was performed (2 segmentectomies, 5 hemihepatectomies, 1 tri-segmentectomy). One patient (a 9-month old girl with a mesenchymal hamartoma, initially diagnosed as hemangioendothelioma by biopsy) first underwent marsupialization, and resection of the recurrence later[21]. Biopsy complications did not occur, and one serious surgical complication was seen. This latter was a stenosis of the left hepatic duct after a right-sided extended hemihepatectomy was performed for a huge mesenchymal hamartoma leading to respiratory insufficiency in a 6 weeks old girl. This stenosis was treated with a Roux-en-Y hepaticojejunostomy but the patient went on to progressive obstructive cholestatic icterus and died 3 months later as a result of liver and respiratory failure. The other 7 patients did well and tumor recurrence had not occurred during last follow-up (median 6 months, range 1-49 months).

Hepatoblastoma (n = 26)/ hepatocellular carcinoma (n = 8)

The characteristics of the 26 patients resected for HB and 8 for HCC (4 had a fibrolamellar type) are shown in Table 1.

Table 1 *Characteristics of children with a hepatoblastoma (HB) or hepatocellular carcinoma (HCC)*

HB	HB	HCC
Total number	26	8
Gender		
Male	9	6
Female	17	2
Median age (months)	16	153
Range	1-100	95-196
No. <3 years	20	0
Median serum a-Fetoprotein (ng/l)	$13x10^4$	5
Range	$62\text{-}4x10^8$	1-25350
PRETEXT staging		
At diagnosis		
Group I	4	0
Group II	12[a]	3
Group III	8	1
Group IV	1[b]	0
NPOC	1[c]	4[d]
Operation		
Segmentectomy	4	1
Hemihepatectomy	11	3
Tri-segmentectomy	11	3
Excision biopsy (follow by OLT)	0	1
No. of patients available for follow-up	21	8
Median follow-up (months)	84	41
Range	3-279	10-127
Died	5	1

NPOC = No pre-operative chemotherapy.

[a] One patient (initially group II) was 'up-staged' and became group III pre-operatively. [b] This patient (initially group IV) was 'down staged' and became group III pre-operatively. [c] Pre-operative PRETEXT group II. [d] Three patients had a fibrolamellar carcinoma, one a HCC by a tyrosinemia type-I.

Serum α-fetoprotein (AFP) was elevated in all patients with HB, and only in 3/8 with HCC. In 13 patients with HB, the serum platelet count was >500x10^9/l. One patient with HCC showed a positive hepatitis A and one a positive hepatitis B serology. Multifocality (≥ 2 tumour nodules) was seen in 6 patients (HB n = 5, HCC n = 1) and initial lung metastases was seen in 9 patients (HB n = 8 (1 with a femur metastasis), HCC n = 1). Biopsies were performed in 23 (68%) patients (HB n = 16, HCC n = 7; true cut n = 16, open n = 7), complicated by a strangulating bowel obstruction in 1 patient. In all cases the diagnosis, established by pre-treatment biopsy, could be confirmed after resection of the tumors in all cases. Primary resection was performed in 1 patient with a HB (for unknown reasons), in 3 patients with a fibrolamellar HCC, and in 1 patient with an HCC by a tyrosinemia type-I despite treatment with 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC). In this latter patient, an excision biopsy of a small HCC in segment VII was performed, followed by an orthotopic liver transplantation (OLT). The other 29 patients underwent surgical resection after chemotherapy. One patient with a HB was 'up-staged' (PRETEXT group II became group III) and one patient with a HB was 'down-staged' (initially unresectable PRETEXT group IV became resectable (group III) after chemotherapy). Thirty patients had histologically confirmed tumor free margins. In the other 4 (1 with a HB, 1 with a HB and lung and femoral metastases, 1 with a HCC, and 1 with a HCC and lung metastases) a local re-resection was performed. No vital tumor cells were found in 2 of the re-resection specimens and all 4 patients received postoperative chemotherapy according to protocol guidelines. Histological classification was possible in 24 of the HB's (Table 2). Surgical complications are shown in Table 3. Re-operation was necessary in 9. Out of the 9 children with lung metastases, 5 underwent a metastasectomy.

Table 2 *Histological findings of patients with a hepatoblastoma*

		Died
Foetal	10	1
Foetal & embryonal	6	0
Foetal with HCC features	1	1
Embryonal	3	1
Embryonal & anaplastic	1	1
Mesenchymal & epithelial	3	1
Impossible	2	0
Total	26	5

HCC : hepatocellular carcinoma.

Table 3 *Surgical complications*

	Biopsy (n = 32)	Resection (n = 48)
Benign tumor (n = 8)		
Bile duct obstruction		1
Hepatoblastoma (n = 26)		
Bile leakage		3
Bleeding		5
Budd-Chiari		1
Fluid collection		1
Strangulating bowel obstruction	1	
Hepatocellular carcinoma (n = 8)		
Bile duct obstruction		2
Slight encephalopathy		1
Other malignancy (n = 6)		0
Total	1	14

Thirty children were available for follow-up. Three with a HB had returned to their homeland in complete remission, 1 with a HB returned to his homeland after a recurrent metastasectomy of the left lung. The 5 children with a HB who died were all PRETEXT group III tumors, 4 of them had initial pulmonary metastases (1 had also femur metastases). The patient with HCC with a positive resection margin and lung metastases died. The remaining 24 patients were in complete remission during last follow-up (the follow-up time is shown in Table 1). A follow-up time of > 5 years was possible in 18 patients (HB n = 14, HCC n = 4). The patient with Budd-Chiari was transplanted 4 years after complete remission had been reached. Cumulative overall survival was 73% (median 7 years) for HB (including the 2 patients who died before surgery and the patient who was unresectable), and 88% (median 3.5 years) for HCC. The relation between PRETEXT staging and overall survival in HB appeared to be significant ($P = 0.003$). Metastases were also significant with overall survival ($P = 0.002$), but other significant variables in relation to survival could not be found (sex: $P = 0.552$, focality: $P = 0.911$, positive resection margin: $P = 0.0514$, re-operation: $P = 0.134$).

Other malignancy (n = 6)

An other liver malignancy than HB or HCC occurred in 6 children (3 males, median age 134 months, range 19-167 months). A RMS occurred in 3 patients and a neuroblastoma, malignant teratoma, and Non-Hodgkin lymphoma (NHL) was seen in 1. Multifocality was only seen in the patient with neuroblastoma and metastases did not occur. PRETEXT staging showed 2 group

II tumors (1 NHL, 1 RMS), 3 group III tumors (2 RMS, 1 malignant teratoma), and a group IV tumor in the patient with a multifocal neuroblastoma. Six biopsies (3 true cut, 3 open) were performed without complications. The patient with a neuroblastoma was initially thought to have a HB (diagnosed on a biopsy), but this was corrected after resection of the tumor. The distinction between a malignant teratoma and a HB could not be made on the biopsy specimen and was made after resection definitively. All 6 patients underwent a resection (1 segmentectomy, 5 tri-segmentectomies (1 with a wedge resection)).

Histologically confirmed tumor free margins were established in all 6 patients treated with chemotherapy according to protocol guidelines at that time followed by surgical resection. One patient with a RMS and the patient with a neuroblastoma died. The patient with NHL underwent surgical resection for a rest tumor in the left liver lobe after complete remission was achieved, and was in complete remission during last follow-up (7 years after surgery). The other 2 patients with a RMS and the patient with a malignant teratoma were in complete remission, 1.5, 7, and 2.5 years after surgery, respectively.

Discussion

The current prognosis of the different malignant liver tumors in children depends on the type of tumor. The prognosis of HB is much better than that of HCC (by chance not in this series, probably due to small numbers and a large extent of resectable tumors) and RMS, probably because of the good sensitivity of HB to pre- and postoperative chemotherapy[3,7,12,13,17,22]. Hepablastoma is mainly a tumor of very young children (median age 16 months) in contrast to HCC, which has a peak incidence between 10 and 14 years[5]. For RMS, 2 age peaks are seen, namely 2-6 years (the embryonal type) and 15-19 years (the alveolar type)[23]. The distinction from HB and HCC can especially be difficult if HB or HCC do not show AFP expression. A good tumor marker for RMS is not available.

Preoperative diagnostic biopsy

In children with HB, consensus exists over the need of a biopsy under the age of 6 months and above the age of 3 years and some authors have suggested to leave out a confirming diagnostic biopsy in a 'pathognomic case' of HB (i.e. a young child between 6 months and 3 years of age who presents with an intrahepatic mass combined with highly elevated serum AFP and thrombocytosis)[5,8,15]. We and other authors recommend a biopsy in all patients if no clear contraindications exist[9,10]. The currently used biopsy techniques show a very low complication rate[24]. We saw only one serious complication after a biopsy (3%) and in all but two cases (1 mesenchymal hamartoma, 1 neuroblastoma) the diagnosis could be confirmed after tumor resection. In one case there was doubt between a HB and a malignant teratoma. This is in line with the experience of the SIOPEL-1 study[9,10], and we think that the low complication rate balances out the change of wrongly administering chemotherapy to patients with a primary liver tumor and an elevated AFP.

Furthermore, in 1997 it was shown already that the borderline of 6 months should be drawn with caution[25]. Although, postnatally the serum AFP level is high and declines until approximately 6 months of age to very low serum levels, there can be still a wide range of serum AFP. In healthy children at 10 months of age, even levels up to 100 ng/ml were measured[25]. A biopsy remains necessary when the AFP level is normal and our data of patients with HB showed that the range of elevated serum AFP level (according to their age) can be broad (62-4x10^8 ng/l). Furthermore, HCC presents in 60-90% of the cases with an elevated AFP as well, and it was suggested that age can help to differentiate between HB and HCC[5]. Although HCC occurs mostly in the older children and adolescents, it has been described in children under the age of 3[26]. Age should be of lesser importance than the condition of the patient to decide whether or not to perform a biopsy. Histology is the only way to be sure about the diagnosis (especially in the 'non-classical' cases) and there is a large difference in prognosis for the different pediatric liver malignancies. Finally, if one wants to study the tumor biology in its primary state, fresh frozen (untreated) tissue is a prerequisite, although an ethical question is raised here.

Surgical results

The 73% survival of HB (median follow-up 7 yrs) in our centre is comparable with other data[8-10], but the 88% survival rate of HCC (median follow-up 3.5 yrs) must be interpreted with caution and must be due to small numbers. Four of the 8 patients with HCC had a fibrolamellar carcinoma. Although it is often described that in children the prognosis of this specific type of HCC is supposed to be better than that of the 'normal' HCC[27-29], this seems not to be true[30]. The worse prognosis for RMS and the good prognosis for Non-Hodgkin lymphoma were confirmed in our analysis[11,31]. This was also the case for the prognostic value of the PRETEXT staging system and metastases in relation to survival[18,32]. Finally, a positive resection margin appeared to be no significant parameter in relation to survival. This raises the question how children with an incomplete resection of their HB (i.e. microscopical residue) should be treated. Remarkably, it was shown before that these children do not have a worse prognosis even if a re-resection is not performed[10]. Hopefully, it will be possible to answer this question in future studies.

Conclusion

In our centre, the treatment results of various pediatric liver tumors with surgical resection and (neo)adjuvant chemotherapy are comparable with those of larger international series except for HCC. The existing diagnostic pitfalls in the differentiation between the various liver malignancies justify and necessitates the use of a diagnostic biopsy. Especially if one considers the safety of the current techniques and the good histological predictive value. Furthermore, fresh frozen (untreated) tumour tissue is a prerequisite to study the tumor biology.

References

1. Parkin DM, Stiller CA, Draper GJ, Bieber CA. The international incidence of childhood cancer. *Int J Cancer* 1988; 42(4):511-20.
2. Mann JR, Kasthuri N, Raafat F, Pincott JR, Parkes SE, Muir KR, et al. Malignant hepatic tumors in children: incidence, clinical features and aetiology. *Paediatr Perinat Epidemiol* 1990; 4(3):276-89.
3. Exelby PR, Filler RM, Grosfeld JL. Liver tumors in children in the particular reference to hepatoblastoma and hepatocellular carcinoma: American Academy of Pediatrics Surgical Section Survey--1974. *J Pediatr Surg* 1975; 10(3):329-37.
4. Reynolds M. Pediatric liver tumors. *Semin Surg Oncol* 1999; 16(2):159-72.
5. Perilongo G, Shafford EA. Liver tumors. *Eur J Cancer* 1999; 35(6):953-8; discussion 958-9.
6. Douglass EC, Green AA, Wrenn E, Champion J, Shipp M, Pratt CB. Effective cisplatin (DDP) based chemotherapy in the treatment of hepatoblastoma. *Med Pediatr Oncol* 1985; 13(4):187-90.
7. Vos A. Primary liver tumors in children. *Eur J Surg Oncol* 1995; 21(1):101-5.
8. Ortega JA, Douglass EC, Feusner JH, Reynolds M, Quinn JJ, Finegold MJ, et al. Randomized comparison of cisplatin/vincristine/fluorouracil and cisplatin/continuous infusion doxorubicin for treatment of pediatric hepatoblastoma: A report from the Children's Cancer Group and the Pediatric Oncology Group. *J Clin Oncol* 2000; 18(14):2665-75.
9. Pritchard J, Brown J, Shafford E, Perilongo G, Brock P, Dicks-Mireaux C, et al. Cisplatin, doxorubicin and delayed surgery for childhood hepatoblastoma a successful approach. Results of the first prospective study of the international society of pediatric oncology (SIOP) – SIOPEL 1. *J Clin Oncol* 2000; 18(22):3819-28.
10. Schnater JM, Aronson DC, Plaschkes J, Perilongo G, Brown J, Otte J-B, et al. Surgical view of the treatment of patients with hepatoblastoma. Results from the first prospective trial of the International Society of Pediatric Oncology Liver Tumor Study Group (SIOPEL-1). *Cancer* 2002; 94(4):1111-20.
11. Otte JB, Aronson D, Vraux H, de Ville de Goyet J, Reding R, Ninane J, et al. Preoperative chemotherapy, major liver resection, and transplantation for primary malignancies in children. *Transplant Proc* 1996; 28(4):2393-4.
12. Czauderna P, Mackinlay G, Perilongo G, Brown J, Shafford E, Aronson D, et al. Hepatocellular carcinoma in children: results of the first prospective study of the international society of pediatric oncology group. *J Clin Oncol* 2002; 20:2798-2804.
13. Katzenstein HM, Krailo MD, Malogolowkin MH, Ortega JA, Lin-Mu Y, Douglass EC, et al. Hepatocellular carcinoma in children and adolescents: results from the Pediatric Oncology Group and the Children's Cancer Group intergroup study. *J Clin Oncol* 2003; 20(12):2789-97.
14. Reynolds M, Douglass EC, Finegold M, Cantor A, Glicksman A. Chemotherapy can convert unresectable hepatoblastoma. *J Pediatr Surg* 1992; 27(8):1080-3; discussion 1083-4.
15. von Schweinitz D, Burger D, Mildenberger H. Is laparatomy the first step in treatment of childhood liver tumors? – The experience from the German Cooperative Pediatric Liver Tumor Study HB-89. *Eur J Pediatr Surg* 1994; 4(2):82-6.
16. von Schweinitz D, Byrd DJ, Hecker H, Weinel P, Bode U, Burger D, et al. Efficiency and toxicity of ifosfamide, cisplatin and doxorubicin in the treatment of childhood hepatoblastoma. Study Committee of the Cooperative Paediatric Liver Tumour Study HB89 of the German Society for Paediatric Oncology and Haematology. *Eur J Cancer* 1997; 33(8):1243-9.
17. Schnater JM, Köhler SE, Lamers WH, Von Schweinitz D, Aronson DC. Where do we stand with hepatoblastoma? a review. *Cancer* 2003; 98(4):668-678.
18. Brown J, Perilongo G, Shafford E, Keeling J, Pritchard J, Brock P, et al. Pretreatment prognostic factors for children with hepatoblastoma-- results from the International Society of Paediatric Oncology (SIOP) study SIOPEL 1. *Eur J Cancer* 2000; 36(11):1418-25.
19. Kaplan EL, Meier P. non-parametric estimation from incomplete observations. *J Amer Statist Ass* 1958; 53:457-481.
20. Kalbfleisch JD, Prentice RL. The statistical analysis of failure time data. New York: J. Wiley, 1980.
21. Meinders AJ, Simons MP, Heij HA, Aronson DC. Mesenchymal hamartoma of the liver: failed management by marsupialization. *J Pediatr Gastroenterol Nutr* 1998; 26(3):353-55.

22. Superina R, Bilik R. Results of liver transplantation in children with unresectable liver tumors. *J Pediatr Surg* 1996; 31(6):835-9.
23. Lanzkowsky P. Rhabdomyosarcoma and other soft tissue sarcomas. In: Willis K, Bowman-Schulman E, eds. Manual of Pediatric Hematology and Oncology, ed. 2nd. New York: Churchill Livingstone, 1995; 453-475.
24. Bravo AA, Sheth SG, Chopra S. Review Articles: Liver Biopsy. *New Engl J Med* 2001; 344(7):495-500.
25. Ohama K, Nagase H, Ogino K, Tsuchida K, Tanaka M, Kubo M, et al. Alpha-fetoprotein (AFP) levels in normal children. *Eur J Pediatr Surg* 1997; 7(5):267-9.
26. Chan KL, Fan ST, Tam PK, Chiang AK, Chan GC, Ha SY. Pediatric hepatoblastoma and hepatocellular carcinoma: retrospective study. *Hong Kong Med J* 2002; 8(1):13-17.
27. Farhi DC, Shikes RH, Murari PJ, Silverberg SG. Hepatocellular carcinoma in young people. *Cancer* 1983; 52(8):1516-25.
28. Barsu M. Fibrolamellar hepatocarcinoma: case report and review of the literature. *Rom J Morphol Embryol* 1991; 37(3-4):161-6.
29. Kearney D, Donlon JB, Mendelson RM. A case of fibrolamellar hepatocellular carcinoma. *Australas Radiol* 1991; 35(1):88-91.
30. Katzenstein HM, Krailo MD, Malogolowkin MH, Ortega JA, Qu W, Douglass EC, et al. Fibrolamellar hepatocellular carcinoma children and adolescents. *Cancer* 2003; 97(8):2006-12.
31. Ramos G, Murao M, de Oliveira BM, de Castro LP, Viana MB. Primary hepatic non-Hodgkin's lymphoma in children: a case report and review of the literature. *Med Pediatr Oncol* 1997; 28(5):370-2.
32. von Schweinitz D, Hecker H, Schmidt-von-Arndt G, Harms D. Prognostic factors and staging systems in childhood hepatoblastoma. *Int J Cancer* 1997; 74(6):593-9.

'Retrospective results must always be interpreted with caution, therefore a prospective trial was designed to evaluate the current value of preoperative chemotherapy in hepatoblastoma'

This has been the first international trial that used neo-adjuvant chemotherapy. Since hepatoblastoma is a tumor with low incidence, the trial was conducted in 91 centers in 33 countries

Chapter 3

Surgical view of the treatment of patients with hepatoblastoma

Results from the first prospective trial of the International Society of Pediatric Oncology Liver Tumor Study Group (SIOPEL-1)

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Abstract

Background. Surgical resection is the cornerstone of treatment for patients with hepatoblastoma (HB). The Society of Pediatric Oncology Liver Tumor Study Group launched its first prospective trial (SIOPEL-1) with the intention to treat all patients with preoperative chemotherapy and delayed surgical resection. The objective of this article was to assess the assumed surgical advantages of primary chemotherapy.

Methods. Between 1990 and 1994, 154 patients age < 16 years with HB were registered on SIOPEL-1. The pretreatment extent of disease was assessed, and, after undergoing biopsy, patients were treated with cisplatin 80 mg/m^2 intravenously over 24 hours and doxorubicin 60 mg/m^2 intravenously over 48 hours by continuous infusion (PLADO). Generally, tumors were resected after four of a total of six courses of PLADO.

Results. One hundred twenty eight patients underwent surgical resection (13 patients underwent primary surgery, and 115 patients underwent delayed surgery after PLADO). A pretreatment surgical biopsy was performed in 96 of 128 patients (75%). Biopsy complications occurred in 7 of 96 patients (7%). Twenty-two patients showed pulmonary metastases at the time of diagnosis, and 7 patients underwent thoracotomy. Operative morbidity and mortality were 18% and 5%, respectively. Complete macroscopic surgical resection was achieved in 106 patients (92%), including 6 patients who underwent orthotopic liver transplantation. The actuarial 5-year event free survival (EFS) rate for all 154 patients in the study was 66% and the overall survival (OS) rate was 75%. For the 115 patients who were included in the surgical analysis that followed the exact protocol, the EFS and OS rates were 75% and 85%, respectively.

Conclusions. Biopsy is a safe procedure and should be performed routinely. Preoperative chemotherapy seems to make tumor resection easier. Reresection of a positive resection margin does not necessarily have to be performed, because postoperative chemotherapy showed good results. Resection of lung metastases can be curative if there is local control of the primary tumor; however, results showed that the patient's prognosis was worse. Surgical morbidity or mortality rates were not necessarily higher in large multicenter studies. More importantly, countries of lesser economic status also can contribute effectively to these trials.

Introduction

There is general agreement that complete surgical resection is the cornerstone of treatment for patients with hepatoblastoma (HB) and hepatocellular carcinoma (HCC) and the only way for eventual cure. HB occurs most frequently in the first few years of life, whereas HCC usually is observed in older children and adolescents[1]. This article deals with HB only.

Historically, only 30% of patients with HB were amenable to primary surgical resection. Currently, with the help of more sophisticated imaging and surgical techniques, the rate is probably closer to 50%. This means that 50% of the tumors are still considered *unresectable* at the time of diagnosis. Half of these neoplasms can be made resectable with modern preoperative chemotherapy. This has been mainly due to the good tumor response of systemic cisplatin (CDDP)-based chemotherapy, which is capable of reducing tumor volume. In other words, eventually, 75% of all tumors can be completely resected[2-11].

The International Society of Pediatric Oncology Liver Tumor Study Group launched its first prospective trial (SIOPEL-1) with the intention of treating all patients with preoperative chemotherapy for the following reasons: 1) The experience of individual surgeons. Compared with the resection of HBs at the time of diagnosis, most surgeons agreed that operating on tumors which had become smaller after chemotherapy was *easier*, and, hence *safer*, because the tumor became better defined, less friable, and less prone to bleeding. 2) Visible metastases were present at the time of diagnosis in 20% of patients; these and micrometastases would be exposed to chemotherapy earlier. In other studies, these patients usually, but not consistently were given preoperative chemotherapy as well. 3) It was desirable to establish a multidisciplinary approach at the onset with the objective of standardizing the selection and clinical grouping of patients, including use of a uniform staging system. For this reason, a *pretreatment extent of disease* (PRETEXT) grouping system was designed specifically for patients with liver tumors (the predictive value of this system will be discussed in more detail in *Chapter IV*). Standardization is particularly important in an international and multi-institutional study of such a rare pediatric tumor. The published overall results of this completed study support the strategy chosen[12-14].

The primary objective of this article was to assess whether the assumed surgical advantages of primary chemotherapy stand up to more detailed scrutiny. Ease and safety of surgery cannot be defined scientifically, so surrogates are needed. These included 1) complications of biopsies, which are prerequisite for preoperative chemotherapy; 2) resectability rate at first attempt; 3) microscopic residual disease; 4) local recurrence rate; and 5) local and systemic complications at surgery and within the first postoperative month.

Material and methods

The SIOPEL-1 study was open to patient registration between January 1990 and February 1994. Ninety-one centers in 33 different countries registered 154 patients with HB age < 16 years. After biopsies had been taken, patients were treated with preoperative chemotherapy. In the protocol a biopsy was recommended in face of unequivocal clinical findings and was mandatory in patients < 6 months and > 3 years because of the increased incidence of other tumor types at these age groups. Biopsy techniques and their complications were analyzed. Small, localized tumors could be treated with primary resection followed by chemotherapy.

Chemotherapy consisted of cisplatin 80 mg/m^2 intravenously over 24 hours on Day 1 and doxorubicin 60 mg/m^2 intravenously over 48 hours by continuous infusion (PLADO) on Days 2 and 3. After four courses, tumor resectability was assessed, and definitive surgery performed if it was considered feasible. Tumor resection was then followed by two more courses of PLADO. If the tumor was still judged to be unresectable after four courses of PLADO and was still responsive to chemotherapy, then two more courses of PLADO were administered, and resectability was assessed again.

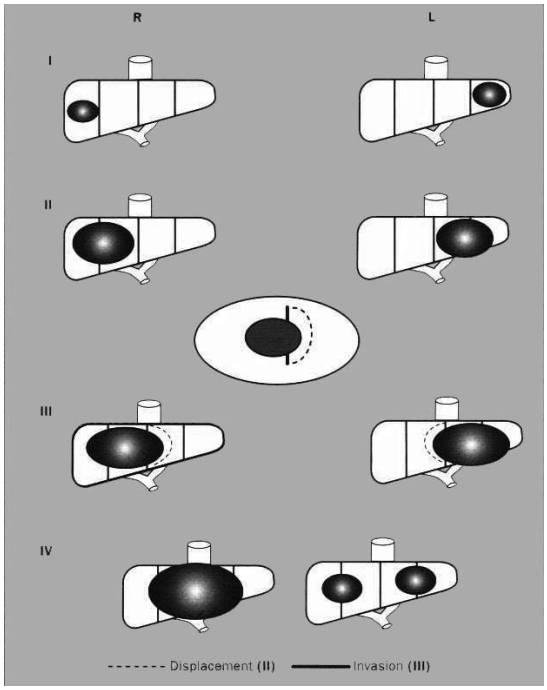
Imaging

The extent of the primary tumor was assessed by abdominal ultrasound with or without Doppler, computed tomography scan, and (optionally) magnetic resonance imaging or hepatic angiography. The original radiological findings were reviewed centrally.

Grouping

A new prechemotherapy and presurgery system based on imaging findings and using the four main liver sectors was used to evaluate tumor extension. It was named the PRETEXT system (Figure 1). The left lobe of the liver consists of a lateral (Couinaud segments 2 and 3) and a medial sector (Couinaud segment 4), and the right lobe is divided in an anterior (Couinaud segment 5 and 8) and a posterior sector (Couinaud segment 6 and 7)[15]. The number of affected liver sector(s) determined the PRETEXT category (Figure 1). Extrahepatic extension was indicated by adding one or more letters as follows: involvement of the hepatic and/or caval vein (V), involvement of the portal vein (P), extrahepatic tumor extension (E), and presence of distant metastases (M). The system was used to assess tumor extent and response to chemotherapy and to determine the optimal timing and type of surgical resection. Its ultimate goal was to ascertain preoperatively whether complete resection of the tumor would be possible.

FIGURE 1 *The pretreatment extent of disease grouping system used for the first prospective trial of the international Society of Pediatric Oncology Liver Tumor Study Group*



Surgery

The following surgical guidelines were recommended: The pretreatment biopsy was a needle biopsy or a wedge resection through a small laparotomy. At least three cores of tissue were taken from different sites. Laparotomy allowed the surgeon to collect more material, to sample different areas, and to control any tumor bleeding. For accurate histologic diagnosis, a generous sample of tumor was taken. Fine-needle biopsy was not accepted.

No specific surgical guidelines were provided for tumor resection, because it would have been futile and misleading given the use of so many techniques. However, to minimize the risk of perioperative deaths, evaluation of the nutritional status of the patients was recommended before surgery, as described by Fan et al.[16], and patients generally were treated in centers with a fairly large experience in liver surgery and anesthesia. Primary surgery was recommended only for those patients with tumors confined to the left lateral sector or the right posterior sector (PRETEXT group I tumors). Usually, after four courses of chemotherapy, partial liver resection was performed if local resectability was feasible, also in the presence of lung metastases. Thus, as soon as local tumor control had been achieved, metastasectomy through either thoracotomy or sternotomy was performed. In patients with unresectable tumors who had lung metastases, chemotherapy was continued to gain maximal tumor response. If reduction of the lung metastasis did not occur, then

they were removed surgically to make the patient eligible for transplantation. A specific guideline for the acceptable amount of tumor free margin was not given as long as no tumor cells were found in the plane of resection.

Orthotopic liver transplantation (OLT) was considered in patients with HB completely confined to the liver that, despite response to adequate first line chemotherapy, remained unresectable. Results of OLT in SIOPEL-1 is presented in *Pediatr Blood Cancer 2004;42:74-83*. In patients with recurrent disease of with persistently elevated α-fetoprotein (AFP) levels, the center had to decide which treatment strategy was best suited for the particular patient.

Results

Patients characteristics

Of the 154 patients who entered the study, 128 were considered eligible for analysis. Twenty-six patients were excluded for the following reasons: six patients died within 16 weeks from the time of diagnosis before surgical resection could be performed; in 16 patients, tumors remained unresectable after PLADO; and, in 4 patients, a complete data set could was not available. Of the 128 patients analyzed, 13 patients underwent primary surgery for various reasons but, on the *intention-to-treat* principle, are included in this analysis. The clinical characteristics are summarized in Table 1.

Table 1 *Characteristics of 128 patients with hepatoblastoma who underwent surgical resection*

Characteristic	No.	%
Gender		
Male	82	64
Female	46	36
Age (yrs)		
Median	1	–
Range	0-13	–
Serum α-fetoprotein (ng/L)		
Median	172,714	–
Range	39-40x10^6	–
Platelet count >500x109/L	77	60
Solitary tumour	102	80
Size at imaging (cm)		
Median	6.8	–
Range	3-17	–
Pulmonary metastases (chest X-ray or lung CT scan)	22	17
PRETEXT group (at diagnosis)		
I	8	6
II	57	45
III	46	36
IV	14	11
Missing	3	2
VPEM positive	21	16
PRETEXT group (at surgery)		
I	6	5
II	55	43
III	47	37
IV	6	5
Missing	13	10
Impossible	1	0
Surgery		
Primary surgery	13	10
Delayed surgery (post PLADO)	115	90
5-years overall survival		
All 154 patients included in SIOPEL 1	116	75
The 115 patients who received delayed surgical resection (post-PLADO)	98	85

CT: computed tomography; PRETEXT: pretreatment extent of disease; VPEM: involvement of the hepatic/caval vein (v), portal vein (p), extrahepatic tumor extension (e), and presence of distant metastases (m); PLADO: cisplatin 80 mg/m^2 over 24 hours on Day 1 and doxorubicin 60 mg/m^2 over 48 hours by continuous, intravenous infusion on Days 2 and 3; SIOPEL-1: the first prospective trial designed by the International Society of Pediatric Oncology Liver Tumor Study Group.

Surgical procedures

Biopsy (n = 96 patients)

Of 128 patients who underwent surgical resection, 96 patients (75%) underwent a pretreatment surgical biopsy. Sixty-three patients underwent closed biopsy (needle biopsy in 20 patients, Tru-Cut biopsy in 37 patients, and other in 6 patients), 30 patients underwent open biopsy, and data about the biopsy technique used was missing in 3 patients. Eighty percent of biopsies were reviewed centrally, with an even spread across all centers, and central review was broadly in agreement with the center's own diagnosis of the primary pathologist. Thirty-two patients did not have a preoperative histologic diagnosis, because clinicians relied on clinical and biochemical characteristics. Complications of biopsy occurred in 7 of 96 patients (7%) (Table 2): bleeding from the biopsy site in 4 patients (1 open biopsy, 3 closed biopsies), abdominal pain in 2 patients (1 open biopsy, 1 closed biopsy), and a wound infection developed in one child who had an open biopsy. All 7 patients recovered completely within hours or a few days.

Table 2 *Surgical complications during or shortly after surgery*

Complication	Biopsy (n = 96)	Primary resection (n=13)	Delayed resection (n=115)
	7 (7%)	**3 (23%)**	**27 (23%)**
Bleeding	4	–	3
Infection	1	2	9
Death	0	1	5
Other	2[a]	–	10[b]

[a] These two patients experienced abdominal pain.

[b] One patient had transient hyperbilirubinaemia; one patient had tubular dysfunction characterized by high urine volume and mild congestive cardiac failure; one patient had duodenal ulceration with perforation; one patients had anesthesia leading to hypothermia and metabolic acidosis; one patient had inflammation and small discharge from wound only; one patient had impaired biliary drainage and portoenterostomy; one patient had hepatomegaly 2 months postresection with clinical jaundice and abnormal liver function tests (biopsy showed portal tract fibrosis, bile duct proliferation, and inflammation; jaundice disappeared but liver function tests remain abnormal); one patient died 18 hours postoperatively of possible myocardial damage (anthracycline related), because there were no technical problems during the operation; one patient had acute liver graft rejection (treated successfully); and one patient had biliary fistula and subphrenic collection later drained percutaneously under computed tomography control.

Primary surgery (n = 13 patients)

Five girls and 8 boys with a median age of 16 months (range, 6 months to 11 years) underwent primary surgery (PRETEXT group I tumors in 4 patients, group II tumors in 6 patients, group III tumors in 2 patients, group IV tumors in no patients, and an unknown group tumor in 1 patient). The reasons for primary surgery were an emergency procedure for bleeding in 2 patients, a pedunculated tumor in 1 patient, and for a small tumor according to protocol guidelines in 1 patient. In

the remaining 9 patients, primary surgery was performed by individual clinical decision despite protocol guidelines.

The median time from the date of diagnosis to resection was 5 days (range, 0-45 days). A left lateral segmentectomy was performed in 1 patient, 5 patients underwent hemihepatectomy (2 right and 3 left), and 4 patients underwent right-sided trisegmentectomy. Nonanatomic resections were performed in 2 patients (one in Couinaud segments 4 and 5 and one in Couinaud segments 5 and 6). In 8 patients, the intraoperative blood loss was < 500 cc (62%); in one patient, blood loss was between 500 cc and 1000 cc (8%); and in 2 patients (15%), blood loss was > 1000 cc. In 2 patients, the amount of intraoperative bleeding was not retrievable from the medical record. Surgery could not be completed in 1 patient because of massive intraoperative bleeding. This patient ultimately died of postsurgical shock the day after surgery. The other 12 underwent complete surgical resection. Two of them developed a wound abscess (Table 2).

The median follow-up of 13 patients who underwent primary surgery was 5.5 years (range, 3-8 years). Twelve patients are alive with no evidence of disease.

Delayed surgery post PLADO (n = 115 patients)

Forty-one girls and 74 boys with a median age of 21 months (range, 4 months to 5 years) underwent surgery after pretreatment with chemotherapy. The median time between the date of diagnosis and the resection was 4 months (range, 2-6 months). Thirty-two of 115 patients (28%) were down-staged after receiving preoperative PLADO. Nine patients initially had group IV (unresectable) tumors and became resectable according to the PRETEXT tumor grouping system. Four patients were *up-staged* (3%). In 1 patient, a resectable tumor (initially, a group II tumor) became unresectable (finally, a group IV tumor). This patient was salvaged with OLT and is in complete remission 58 months after transplantation (the predictive value of the PRETEXT grouping system is reviewed in detail in *Chapter IV*).

A left lateral segmentectomy was performed in 5 patients, 26 patients underwent right hemihepatectomy (5 including Couinaud segment 1), 12 patients underwent left hemihepatectomy (1 with Couinaud segment 1 and 1 with a part of Couinaud segment 5), 26 patients underwent right trisegmentectomy (11 with Couinaud segment 1), and 9 patients underwent left trisegmentectomy (4 with Couinaud segment 1). In 15 patients, an extra-anatomic resection was performed (5 with Couinaud segment 1, 6 with Couinaud segment 2, and 4 with Couinaud segment 3). Six patients (5%) underwent primary transplantation (OLT; these patients are reviewed in *Pediatr Blood Cancer 2004;42:74-83*). Complete data sets could not be obtained in 16 of 115 patients (14%) who underwent delayed surgery post-PLADO. Intraoperative bleeding was < 500 cc in 52 patients (45%), between 500 cc and 1000 cc in 26 patients (23%), and > 1000 cc in 11 patients (10%). In 26 patients (23%), the amount of intraoperative bleeding was not recorded. There was wide variety in the techniques used for surgical resection. In 40 patients, finger fracture was applied; in 31 patients, ultrasonic dissection with the Cavitron Ultrasonic Surgical Aspirator (CUSA) was used; and, in 9 patients, resection was performed with (Bovie) electrical coagulation. One patient underwent resection with vascular bypass. In 34 patients, the technique was not

documented. Twenty-seven of 115 patients (23%) developed a surgical complication (Table 2). There was no clear link between advanced PRETEXT category and surgical morbidity/mortality (data not shown).

Five patients died perioperatively (Table 3), and, macroscopically, 4 tumors were resected incompletely; thus, successful, complete surgical resection was achieved in 106 patients (92%), including 6 patients who underwent OLT. Microscopic assessment revealed tumor present at margins of surgical resection in 11 patients, and 4 patients showed tumor present in main vein (for details, see Table 4).

Table 3 *Characteristics of six patients who died of surgery related causes*

Gender	Age (months)	PRETEXT group	LM	CR	Resection	Major technique	Cause of death
Primary resection (n = 13 patients)							
M	12	III	–	+	Not completed	FF	Postsurgical shock
Delayed resection (n = 115 patients)							
F	27	II	–	+	R hemi	FF	Cardiac arrest at operation
M	19	II	–	+	Unknown	Unknown	Bleeding
F	8	III (m)	+	+	L hemi	FF	Bleeding postsurgery; cardiac arrest at second look
F	31	II (m)	+	+	R hemi	FF	Kinking of hepatic artery with total necrosis of OLT 6 days after OLT
M	24	III (vpe)	–	–	R trisegm	FF and diathermy	Bleeding during surgery; died shortly after surgery

PRETEXT: pretreatment extent of disease; LM: lung metastases; CR: complete resection; M: male; FF: finger fraction; F: female; R: right; hemi: hemihepatectomy; m: presence of distant metastases; L: left; OLT: orthotopic liver transplantation; vpe: involvement of the hepatic/caval vein (v), portal vein (p), and extrahepatic tumor extension (e); trisegm: trisegmentectomy

TABLE 4 *Characteristics of 15 patients with microscopical residual disease who underwent delayed surgical resection*

Gender	Age (months)	PRETEXTgroup	LM	Resection	Major Technique	RM	MVTP	Follow-up
F	13	III	–	Local excision	FF and diathermy	+	–	NED
M	19	III	–	L hemi	FF	+	–	NED
F	4	III	–	R trisegm	CUSA	+	–	NED
M	8	III (vpm)	+	L hemi	CUSA	+	–	NED
F	8	III (v)	–	R trisegm	FF and diathermy	+	–	NED
M	34	III	–	L trisegm	CUSA	+	–	Died
M	155	III	–	R trisegm	CUSA	+	–	NED
F	16	III (vpe)	–	R hemi	CUSA and diathermy	+	–	Died
M	0	II	–	L hemi	FF and CUSA	+	–	NED
F	46	II (pm)	–	R hemi	CUSA and diathermy	–	+	Lost
M	16	IV	–	R hemi	CUSA and diathermy	+	–	NED
F	91	III (vp)	–	L trisegm	FF	–	+	NED
M	4	III	–	L trisegm	Unknown	+	–	NED
M	11	II	–	Local excision	Other	–	+	NED
F	9	II	–	L hemi	CUSA	–	+	NED

PRETEXT: pretreatment extent of disease; LM: lung metastases; RM: resection margin; MVTP: tumor present at main vein; F: female; FF: finger fraction; NED: no evidence of disease; M: male; L: left; hemi: hemi-hepatectomy; R: right; trisegm: trisegmentectomy; CUSA: Cavitron ultrasonic surgical aspirator; vpme: involvement of the hepatic/caval vein (v), portal vein (p), distant metastases (m), and extrahepatic tumor extension (e).

In 9 of 11 patients with microscopic tumor present at margins of surgical resection, the resection had been performed using the CUSA. Two of 15 patients residual disease died (1 patient with progressive disease and 1 patient because of Budd-Chiari syndrome), 1 patient was treated with local radiotherapy and survived without evidence of disease (follow-up 6 years), 1 patient with tumor present in the main vein was lost to follow-up, and all other patients had no evidence of disease at a mean follow-up of 5.5 years (range, 2-8 years). Nine patients were treated with postoperative chemotherapy. In one patient with microscopic residual disease, data regarding their postsurgical treatment were missing.

The median follow-up of all patients who underwent tumor resection after PLADO was 4.5 years (range, 1-8 years). Three patients were lost to follow-up. The 5-year overall survival (OS) rate and the 5-year event free survival (EFS) rate for all 154 patients who were included in the study was 75% (95% confidence interval [95% CI], 68-82%) and 66% (95% CI, 59-74%) respectively[14]. The OS and EFS rate (with 95% CI) for the 115 patients included in this surgical analysis who followed the exact protocol were 85% (95% CI, 78-92%) and 75% (95% CI, 67-83%), respectively (Figs. 2, 3).

Figure 2 *The 5-year overall survival of patients who underwent delayed surgery (n = 115 patients) after receiving PLADO*

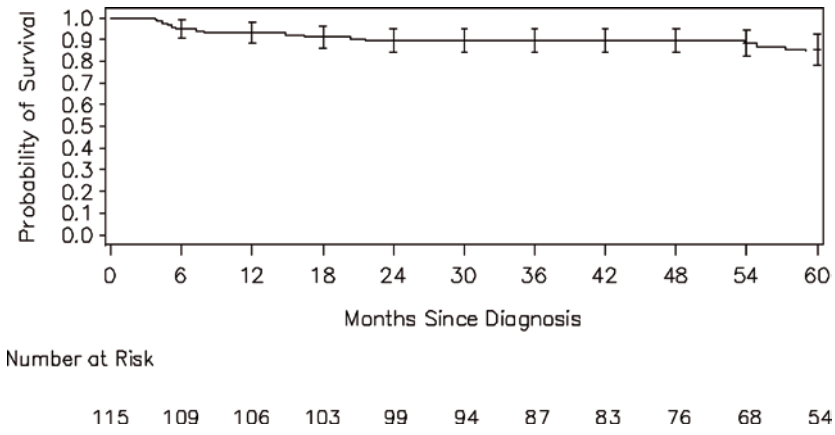
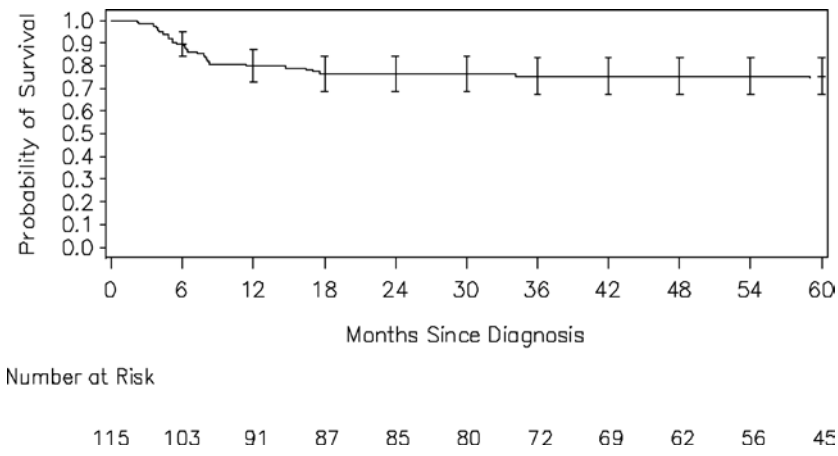


Figure 3 *The 5-year event free survival of patients who underwent delayed surgery (n = 115 patients) after receiving PLADO*



Mortality and recurrence

Sixteen patients died (13%) (Table 3). One patient with a PRETEXT group III tumor died during primary surgery, and 5 patients died during or after delayed surgery (2 patients with group II tumors and 3 patients with group III tumors); thus, the overall surgical mortality rate was 5% (6 of 128 patients). Five of these patients underwent hepatic resection using the finger-fracture technique (1 patient with diathermy). Data on the operative technique used in the sixth patient were not available. The remaining 10 patients died of disease-related causes.

Only 2 of 16 patients who died (13%) had microscopic residual disease after surgery. One patient died 7 months postoperatively of Budd-Chiari syndrome after local recurrence (tumor-related death), because, after undergoing a right hemihepatectomy, the left hepatic vein became occluded by tumor. AFP levels remained elevated in the other patient, but the exact time between the diagnosis and the date of death could not been traced, because he was lost to follow-up.

Ten patients (8%) experienced disease recurrence (PRETEXT grouping II tumors in 3 patients, group III tumors in 5 patients, and group IV tumors in 2 patients) after undergoing apparently complete resection: 5 patients developed a local recurrence, and 5 patients developed a distant recurrence in the lung. Of the 5 children with a local recurrence, 2 children had lung metastases at the time of diagnosis. Of the 5 children who had a recurrence in the lung, 3 children had lung metastases at the time of diagnosis. None of these children had positive resection margins at first resection. One of them was treated with primary surgery. The primary tumor resections were performed with finger fracture (n = 3 patients), electrocoagulation (n = 3 patients), or CUSA (n = 2 patients). In 2 patients, details of the technique could not be found. The 5 patients with local recurrences underwent surgery: all patients were reresected completely, 2 patients underwent surgery combined with chemotherapy, and 1 patient underwent combined surgery with chemotherapy and radiotherapy. Two of those patients showed no evidence of disease of the most recent follow-up, 24 months and 84 months after the recurrence, respectively. One patient died during surgery due to uncontrollable bleeding. One patient developed a second recurrence then a third recurrence, underwent transplantation, and finally died. Another patient experienced four recurrences. The first 3 recurrences were treated with resection, and the last recurrence was treated with chemotherapy. Follow-up at 48 months after the first resection showed evidence of disease, and the patient underwent OLT 4 months later. The patient was alive without evidence of disease at last follow-up, 5.5 years from the time of diagnosis.

The 5 patients who developed recurrent disease in the lungs underwent metastasectomy followed by chemotherapy for local control (n = 2 patients, see *Thoracotomies*) or with chemotherapy alone (n = 3 patients). A second recurrence occurred in 2 patients. The first patient was treated with chemotherapy alone but developed recurrent disease 12 months postoperatively and was treated again with chemotherapy alone. The second patient developed a recurrence 13 months postoperatively and was treated with surgery and chemotherapy. Both children were in complete remission at follow-up 3 years and 5 years after their recurrence. Of the other 3 children, 2 children showed no evidence of disease at 3 years and 5 years of follow up, respectively. One child died 6 years after undergoing OLT.

Thoracotomies (n = 7 procedures in 4 patients)

Of 22 patients with lung metastases at the time of diagnosis, 2 patients underwent single metastasectomy. Complications did not occur, and neither patient showed evidence of disease at follow-up 2.5 years and 7 years after resection. Two patients underwent multiple thoracotomies for recurrence in the lungs (2 metastasectomies in one patient and 3 metastasectomies in another patient) without complications. These patients were free of disease with relatively long follow-up (3 and 5 years after resection). Of the remaining 18 patients, 5 patients who were treated with chemotherapy only also were long-term survivors (median follow-up, 6 years; range, 3-7 years). Thus, in total, 9 of 22 patients (41%) were long-term survivors, and 4 of them underwent metastasectomies as well as chemotherapy[13].

Discussion

This article reports the surgical details of patients with HB who were treated according to the SIOPEL-1 protocol based on preoperative PLADO chemotherapy. The demographic characteristics of the patients in SIOPEL-1 are comparable with other studies on treating HB. The tumor occurred mostly in younger children, with a male predominance, and, in most patients, serum AFP levels and platelet counts were highly elevated[1,2,6,17]. The vast majority occurred as a solitary tumor in the right lobe of the liver, and, in 22 of 128 patients (17%), there was pulmonary metastatic spread.

Biopsy

Appeared to be a safe procedure with a low complication rate. Only 7% of patients developed a minor complication, a rate comparable with data in the literature[10]. Tumor spill or implantation metastases, which have been reported after biopsies for patients with HCC[18,19], did not occur. The need for biopsy when imaging findings are characteristic and serum AFP levels are elevated remains a controversial matter among surgeons, at least in Europe. We conclude that the low complication rate in SIOPEL-1 justifies the use of biopsies. Malignant primary germ cell tumors of the liver and HCC can be excluded and, in the future, unfavorable histologic features, like undifferentiated HB may be used direct treatment to a high-risk regimen. Furthermore, biopsy material (untreated tumor tissue) is becoming more and more important in molecular biologic studies of tumor markers and for studying tumor biology.

Effect of pre-operative chemotherapy on surgery

This study has shown the advantages of treating patients with PLADO before they undergo tumor resection. First and foremost, excluding the patients who underwent OLT, it was possible to resect the tumor completely in 100 of 115 patients (87%) who were treated with chemotherapy. This emphasizes the fact that modern treatment strategies based on effective chemotherapy regimens have dramatically improved the complete resection rate if we keep in mind that historically, only 30%

of patients were eligible for complete tumor resection. Furthermore, the OS rate has improved from 35% in the early 1970s to the current rate of 70-75%[2,11,14].

It often has been commented by surgeons that tumor resection is '*easier*' after chemotherapy. The neoplasm becomes more solid, less prone to bleeding, and more demarcated from the surrounding healthy liver parenchyma. In comparing the data on intraoperative blood loss between patients who underwent primary surgical resection and patients who underwent delayed surgical resection, this opinion could not be confirmed: The data were more or less equal. However, 28% of patients were *down-staged* after receiving preoperative PLADO. In addition, 9 patients with initially unresectable (group IV) tumors became resectable according to the PRETEXT system. In these patients, a smaller and, thus, easier resection could be performed as a result of the preoperative chemotherapy. The disadvantage of the delay in resection due to preoperative chemotherapy was also shown, although in a smaller group of patients. Four patients (3%) were *up-staged*, and, in 1 patient, a resectable tumor (initially, PRETEXT group II) even became unresectable (finally, PRETEXT group IV).

Surgical procedures

Showed an overall morbidity rate of 18% (24 of 128 patients) and a surgical mortality rate of 5% (6 of 128 patients). Thus, although preoperative chemotherapy may make more tumors resectable, hepatic surgery remains difficult, with definitive morbidity and mortality. An experienced surgical team, therefore, should perform hepatic resections in children. Furthermore, results may be influenced by the design of the study, in which centers with different levels of experience with liver surgery that were located in countries with different economic status participated. Patient selection was not equal in all centers, and this eventual bias may have contributed to the differences found.

Positive resection margins

The favorable outcome for those patients was a notable finding in this series. Of 11 patients with positive margins, only 2 died, but neither of those 2 patients had a local recurrence. None of the 11 patients underwent second resection, and all but 1 patient (radiotherapy) were treated only with postoperative chemotherapy. There were no recurrences, and all survivors were in complete remission at their last follow-up (mean, 5.5 years). This shows that reresection of the positive margin may not necessarily have to be performed. These favorable results may be explained in most patients by the use of the CUSA. The resection margin is '*vacuum cleaned*' and, thus, ablated. Hypothetically, it is possible that, despite the positive margin at the specimen site, there may have been a negative margin at the patient's site. Alternatively or in addition, residual tumor may not be viable because of lethal damage from preoperative chemotherapy.

Surgical resection of pulmonary metastases

Seems a good treatment option if local control of the liver tumor has been accomplished. All 4 of the 22 patients with pulmonary metastases at the time of diagnosis who underwent a metastasectomy survived without residual disease. Even when there was a recurrence, surgical treatment was a curative procedure as long as there was local control of the primary tumor. Micrometastases at the time of diagnosis obviously are treated by the preoperative chemotherapy.

In summary, our data show that biopsy is a safe procedure with a low complication rate, and we still recommend it in our current trials. Not only does it provide an exact histologic diagnosis; in a treatment strategy based on the use of preoperative chemotherapy, it also is the only way to learn more about the histologic variants of hepatoblastoma and their biologic characteristics. If this issue is not addressed systematically within the context of a well-defined study, then the risk will be run that potential crucial information will be lost that may rectify and improve the actual treatment results. The use of a treatment strategy based on preoperative chemotherapy and delayed surgery for patients with such chemosensitive tumors as hepatoblastoma, whose response to therapy can be monitored easily, is at least as effective as strategies based on primary surgery. From a surgical perspective, it seems that preoperative treatment makes resection easier without any relevant drawback for the patients. Surgical morbidity and mortality rates of 18% and 5%, respectively, are comparable with the rates cited in the literature and support the chosen strategy[6, 17, 20]. If a positive resection margin is noted by the pathologist, then reresection does not necessarily have to be performed. The treatment with postoperative chemotherapy or radiotherapy showed good results, and these patients did not experience local disease recurrence. Although the overall prognosis for patients with lung metastases is worse, resection of lung metastases may be curative if local tumor control has been achieved. Finally, the results show that large, multicenter studies do not necessarily lead to higher surgical morbidity or mortality rates. More importantly, the results have shown that countries of lesser economic status can contribute crucially to these trials.

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References

1. Vos A. Primary liver tumours in children. *Eur J Surg Oncol* 1995; 21(1):101-5.
2. Exelby PR, Filler RM, Grosfeld JL. Liver tumors in children in the particular reference to hepatoblastoma and hepatocellular carcinoma: American Academy of Pediatrics Surgical Section Survey – 1974. *J Pediatr Surg* 1975; 10(3):329-37.
3. Douglass EC, Green AA, Wrenn E, Champion J, Shipp M, Pratt CB. Effective cisplatin (DDP) based chemotherapy in the treatment of hepatoblastoma. *Med Pediatr Oncol* 1985; 13(4):187-90.
4. Evans AE, Land VJ, Newton WA, Randolph JG, Sather HN, Tefft M. Combination chemotherapy (vincristine, adriamycin, cyclophosphamide, and 5-fluorouracil) in the treatment of children with malignant hepatoma. *Cancer* 1982; 50(5):821-6.
5. Ortega JA, Krailo MD, Haas JE, King DR, Ablin AR, Quinn JJ, et al. Effective treatment of unresectable or metastatic hepatoblastoma with cisplatin and continuous infusion doxorubicin chemotherapy: a report from the Childrens Cancer Study Group. *J Clin Oncol* 1991; 9(12):2167-76.
6. Reynolds M, Douglass EC, Finegold M, Cantor A, Glicksman A. Chemotherapy can convert unresectable hepatoblastoma. *J Pediatr Surg* 1992; 27(8):1080-3; discussion 1083-4.
7. Burger D, von Schweinitz D, Weinel P, Mildenberger H. HB89: the liver tumor study of the Society for Paediatric Oncology. An interim report. *Chirurg* 1992; 63(3):193-8.
8. Weinblatt ME, Siegel SE, Siegel MM, Stanley P, Weitzman JJ. Preoperative chemotherapy for unresectable primary hepatic malignancies in children. *Cancer* 1982; 50(6):1061-4.
9. Filler RM, Ehrlich PF, Greenberg ML, Babyn PS. Preoperative chemotherapy in hepatoblastoma. *Surgery* 1991; 110(4):591-6; discussion 596-7.
10. von Schweinitz D, Burger D, Mildenberger H. Is laparatomy the first step in treatment of childhood liver tumors? – The experience from the German Cooperative Pediatric Liver Tumor Study HB-89. *Eur J Pediatr Surg* 1994; 4(2):82-6.
11. von Schweinitz D, Byrd DJ, Hecker H, Weinel P, Bode U, Burger D, et al. Efficiency and toxicity of ifosfamide, cisplatin and doxorubicin in the treatment of childhood hepatoblastoma. Study Committee of the Cooperative Paediatric Liver Tumour Study HB89 of the German Society for Paediatric Oncology and Haematology. *Eur J Cancer* 1997; 33(8):1243-9.
12. Brown J, Perilongo G, Shafford E, Keeling J, Pritchard J, Brock P, et al. Pretreatment prognostic factors for children with hepatoblastoma – results from the International Society of Paediatric Oncology (SIOP) study SIOPEL 1. *Eur J Cancer* 2000; 36(11):1418-25.
13. Perilongo G, Brown J, Shafford E, Brock P, De Camargo B, Keeling JW, et al. Hepatoblastoma presenting with lung metastases. *Cancer* 2000; 89(8):1845-53.
14. Pritchard J, Brown J, Shafford E, Perilongo G, Brock P, Dicks-Mireaux C, et al. Cisplatin, doxorubicin and delayed surgery for childhood hepatoblastoma a successful approach. Results of the first prospective study of the international society of pediatric oncology (SIOP) - SIOPEL 1. *J Clin Oncol* 2000; 18(22):3819-28.
15. Couinaud C. Lobes et segments hepatiques. *Presse Med* 1954; 62:709-12.
16. Fan ST, Lo CM, Lai EC, Chu KM, Liu CL, Wong J. Perioperative nutritional support in patients undergoing hepatectomy for hepatocellular carcinoma. *N Engl J Med* 1994; 331(23):1547-52.
17. von Schweinitz D, Hecker H, Burger D, Mildenberger H. Surgical therapy of hepatoblastoma in childhood. *Langenbecks Arch Chir* 1995; 380(6):315-20.
18. Smith EH. Complications of percutaneous abdominal fine-needle biopsy. Review. *Radiology* 1991; 178(1):253-8.
19. Schotman SN, De Man RA, Stoker J, Zondervan PE, Ijzermans JN. Subcutaneous seeding of hepatocellular carcinoma after percutaneous needle biopsy. *Gut* 1999; 45(4):626-7.
20. King DR, Ortega J, Campbell J, Haas J, Ablin A, Lloyd D, et al. The surgical management of children with incompletely resected hepatic cancer is facilitated by intensive chemotherapy. *J Pediatr Surg* 1991; 26(9):1074-80; discussion 1080-1.

'A new staging system is only valuable if its predictive value is comparable with other well-known staging systems'

Chapter 4

Predictive value of the pretreatment extent of disease system in hepatoblastoma

Results from the International Society of Pediatric Oncology Liver Tumor Study Group (SIOPEL-1)

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Abstract

Background. Preoperative staging (pretreatment extent of disease [PRETEXT]) was developed for the first prospective liver tumor study by the International Society of Pediatric Oncology (SIOPEL-1 study; preoperative chemotherapy and delayed surgery). Study aims were to analyze the accuracy and interobserver agreement of PRETEXT and to compare the predictive impact of three currently used staging systems.

Methods. Hepatoblastoma (HB) patients younger than 16 years who underwent surgical resection (128 of 154 patients) were analyzed. The centrally reviewed preoperative staging was compared with postoperative pathology (accuracy) in 91 patients (81%), and the local center staging was compared with the central review (interobserver agreement) in 97 patients (86%), using the agreement beyond change method (weighted κ). The predictive values of the three staging systems were compared in 110 patients (97%) using survival curves and Cox proportional hazards ratio estimates.

Results. Preoperative PRETEXT staging compared with pathology was correct in 51%, overstaged in 37%, and understaged in 12% of patients (weighted $\kappa = 0.44$; 95% CI, 0.26 to 0.62). The weighted κ value of the interobserver agreement was 0.76 (95% CI, 0.64 to 0.88). The Children's Cancer Study Group/Pediatric Oncology Group-based staging system showed no predictive value for survival ($P = 0.516$), but the tumor-node-metastasis-based system and PRETEXT system showed good predictive values ($P = 0.0021$ and $P = 0.0006$, respectively). PRETEXT seemed to be superior in the statistical fit.

Conclusions. PRETEXT has moderate accuracy with a tendency to overstage patients, shows good interobserver agreement (reproducibility), shows superior predictive value for survival, offers the opportunity to monitor the effect of preoperative therapy, and can also be applied in patients who have not had operations. For comparability reasons, we recommend that all HB patients included in trials also be staged according to PRETEXT.

Introduction

Hepatoblastoma (HB) is the most common malignant liver tumor in children[1]. In recent years, its prognosis has improved dramatically because of combined treatment strategies that used cisplatin-based chemotherapy combined with surgery, as shown in several studies[2-4]. The first prospective study that was launched by the Liver Tumor Study Group of the International Society of Pediatric Oncology (SIOP), known as SIOPEL-1, combined preoperative cisplatin with doxorubicin (PLADO) followed by surgical resection. All patients were treated with preoperative chemotherapy to reduce the size of the tumor, improve the success of resection, and treat microscopic metastases. This resulted in a 5-year overall survival rate of 75% in SIOPEL-1, and new study protocols to improve these results (SIOPEL-2 , and SIOPEL 3) were designed[5-9].

In the SIOPEL-1 prospective trial, a preoperative surgical staging system, the pretreatment extent of disease (PRETEXT) system, which was based on the anatomy of the liver, was developed and adopted[10,11]. The main difference from other well-known liver tumor staging systems, such as the tumor-node-metastasis (TNM) system of the International Union Against Cancer and the system used by the Children's Cancer Study Group (CCSG) and the Pediatric Oncology Group (POG)[2,4,12], is that the PRETEXT system was especially developed to compare the efficacy of various chemotherapeutic regimens in HB and to stage the tumor *before* surgical treatment, whereas the other two systems stage the tumor *post*operatively. PRETEXT was used as a relatively objective but noninvasive method to assess tumor extent at diagnosis and subsequent chemotherapy response and to determine the optimal time and type of resection. Its ultimate goal was to ascertain preoperatively whether it would be possible to perform a radical resection.

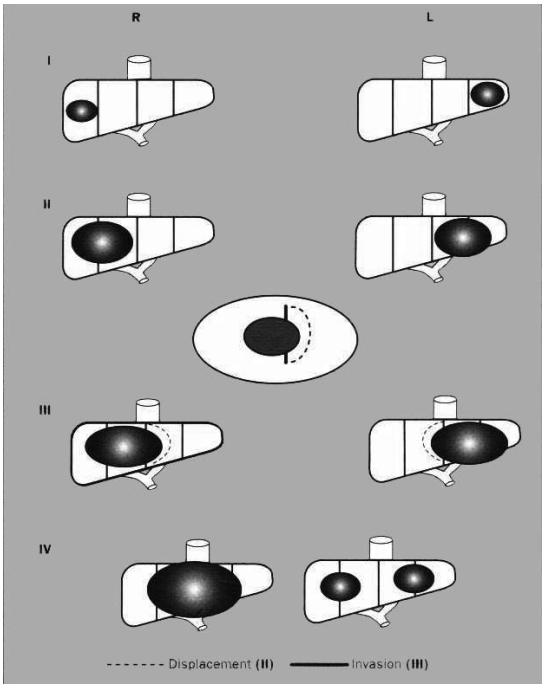
In 1997 Von Schweinitz *et al* investigated the predictive impact of the different staging systems (mentioned in the previous paragraph) in 72 patients treated in the German Pediatric Liver Tumor Study HB89 and proposed using the TNM system to compare treatment results in HB[13]. The aims of the SIOPEL-1 study group in this article were to evaluate the accuracy of the PRETEXT staging system against surgery ('*gold standard*'), to study the interobserver agreement of PRETEXT, and to compare the predictive values of the different staging systems among patients who underwent delayed surgical resection of their tumor and subsequently followed the SIOPEL-1 protocol[7].

Material and methods

PRETEXT staging system

The PRETEXT system, which is based exclusively on imaging at diagnosis and, thus *before* (surgical) therapy, divides the liver into four parts, called sectors. The left lobe of the liver consists of a lateral (Couinaud segments 2 and 3) and a medial sector (segment 4), whereas the right lobe is divided into an anterior (segments 5 and 8) and a posterior sector (segments 6 and 7)[11,14]. Couinaud segment 1 is identical with caudate lobe and is not included in this division. The tumor is classified into one of the following four PRETEXT categories depending on the number of liver sectors free of tumor (Figure 1). PRETEXT I, three adjacent sectors free of tumor; PRETEXT II, two adjacent sectors free of tumor (or one sector in each hemi-liver); PRETEXT III, one sector free of tumor (or two sectors in one hemi-liver and one nonadjacent sector in the other hemi-liver); and PRETEXT IV no tumor-free sectors. Extrahepatic growth is indicated by adding one or more of the following characters: V, vena cava and/ or main tributaries (caval attachments); P, portal vein and/ or main tributaries (hilar); E, extrahepatic excluding extrahepatic V or P (rare); and M,

Figure 1 *The Liver Tumor Study Group of the International Society of Pediatric Oncology (SIOP) SIOPEL-1 pretreatment extent of disease grouping system (PRETEXT)*



distant metastases (mostly lungs, otherwise specify). The assessment of the extent of the primary tumor is performed by abdominal ultrasound and computer tomography (CT). Magnetic resonance imaging or hepatic angiography is only performed if thought necessary by the local center. A lung CT scan is indicated to assess metastatic spread only if the chest X-ray is suspect.

Patients were staged according to the PRETEXT system at diagnosis, during neoadjuvant chemotherapy, and before surgery. The original radiological films were centrally reviewed by one radiologist (C.R.S.). For the comparison study between PRETEXT and pathology, the postchemotherapy PRETEXT taken before surgery was used.

Patients

Between January 1990 and February 1994, patients younger than 16 years old with HB were registered onto the SIOPEL-1 study. See Brown *et al* and Pritchard *et al* for a detailed description of study design, data collection, and definitions of event-free survival and overall survival[5,6]. In short, all patients were treated preoperatively with PLADO after a biopsy had been taken according to the intention-to-treat principle. In case of unequivocal clinical findings, a biopsy was recommended but was mandatory in patients aged less than 6 months and more than 3 years because of the increased prevalence of other tumor types in these age groups. After four courses of PLADO, tumor resectability was assessed by imaging, and definitive surgery was performed if considered feasible. Tumor resection was then followed by two more courses of PLADO. Orthotopic liver transplantation (OLT) was considered in patients with HB in all four liver sectors but completely confined to the liver, despite a positive response to adequate first-line chemotherapy. Results of OLT in SIOPEL-1 is reported in *Pediatr Blood Cancer* 2004;42:74-83. A total of 154 patients from 91 centers in 33 different countries entered onto the study, 128 of whom underwent resection of their primary liver tumor according to protocol guidelines[7]. Of these 128 patients, 15 patients had no central review of their preoperative PRETEXT. Thus, this comparative study focuses on the subset of the remaining 113 patients who all had a centrally reviewed preoperative PRETEXT and who all underwent surgery.

Accuracy and interobserver agreement of PRETEXT

To evaluate the accuracy of the PRETEXT system, results from the PRETEXT system taken *after* chemotherapy and *before* surgery were compared with results from the pathology report of the operative specimen using agreement beyond change (weighted κ). A weighted κ is a κ calculated with different weights that were given to the disagreements according to the magnitude of the discrepancy. For this purpose, the postoperative staging ('*gold standard*') derived from the pathology report was retrospectively performed by doctors who were unaware of the PRETEXT results (J.M.S. and D.C.A.). The weighted κ was also calculated to evaluate the interobserver agreement by comparing PRETEXT staging results obtained from the local center with those from the central review.

Other staging systems

The CCSG/POG staging system and the conventional TNM system for (adult) liver carcinomas were retrospectively applied to the patients after the pathology report was available. The staging was performed in a blinded fashion, with the PRETEXT staging (at diagnosis) of that tumor being unknown (J.M.S. and D.C.A.). The CCSG/POG system distinguishes the following four disease stages: stage I, complete surgical resection; stage II, microscopic residual disease; stage III, macroscopic residual disease; and stage IV, metastatic spread[2,4]. In the TNM system, the T status comprises tumor size (≤ or >2cm), vascular invasion, lobe involvement, multifocality of tumor nodes, and extrahepatic growth; the N status records involvement of lymph nodes; and the M status distant metastases[12,15]. The different TNM stages are listed in Table 1. We are aware that, in contrast to the PRETEXT staging system, the CCSG/POG staging system and TNM system are postoperative staging systems that are validated on the surgical results before any other therapeutic intervention and that now they are being applied to patients who have been pretreated with chemotherapy.

Table 1 *Tumor-node-metastasis (TNM) system for (adult) liver carcinomas*[12,15]

Stage	Group	Description	
I	T1 N0 M0	T1:	solitary tumor, ≤2 cm, without vascular invasion
		N0:	no regional lymph node metastasis
		M0:	no distant metastasis
II	T2 N0 M0	T2:	solitary tumor, ≤2 cm, with vascular invasion;
			or multiple tumors, ≤2 cm, limited to one lobe without vascular invasion;
			or solitary tumor, >2 cm, without vascular invasion
IIIA	T3 N0 M0	T3:	solitary tumor, >2 cm, with vascular invasion;
			or multiple tumors, ≤2 cm, limited to one lobe with vascular invasion
			or multiple tumors, >2 cm, limited to one lobe with or without vascular invasion
IIIB	T1 N1 M0	N1:	regional lymph node metastasis
		T2 N1 M0	
		T3 N1 M0	
IVA	T4 each N M0	T4:	multiple tumors in more then one lobe;
			or ingrowth of tumor(s) in portal or hepatic vein(s);
			or ingrowth in adjacent organs other than the gallbladder;
			or perforation of the visceral peritoneum
IVB	any T any N M1	M1:	distant metastasis

Comparison and survival analysis

The predictive values of the three different staging systems were compared using the Akaike information criterion (AIC) obtained from each of the Cox proportional hazards models. The AIC (= -2ln [maximum likelihood] + 2 [number of fitted parameters]) is a descriptive statistic only and not a formal hypothesis test. It provides a useful measure for comparing different models[16]. Subsequent overall survival curves of the different staging systems were obtained with the Kaplan-Meier method and compared within each system with the log-rank test[17,18]. Overall survival was defined as the time interval between the date of diagnosis and the date of death (from any cause) or the date of last follow-up. The level of significance was considered $P < 0.05$. Statistical procedures were performed with the SAS statistical package version 8.02 (SAS institute, Cary, NC).

Results

Centrally reviewed preoperative PRETEXT staging was available in all 113 patients. The patient characteristics are listed in Table 2. Median age at diagnosis was 17 months (range, 1-155 months), and median follow-up time was 5 years (range, 0-99 months). In 89 (79%) of 113 patients, a biopsy was performed. In the remaining 24 patients, the clinical diagnosis of HB was confirmed in the operative specimen. According to the protocol (suspicion on chest X-ray), 87 (77%) of 113 patients had a CT scan of the chest, and 20 patients (18%) had lung metastases at time of diagnosis. The frequency of the centrally reviewed preoperative PRETEXT stages were as follows: group I, 13 patients (12%); group II, 64 patients (57%); group III, 31 patients (27%), and group IV, 5 patients (4%).

Table 2 *Patient characteristics of 113 patients with HB who entered the SIOPEL-1 study, underwent surgical resection, and had a centrally reviewed preoperative PRETEXT staging*

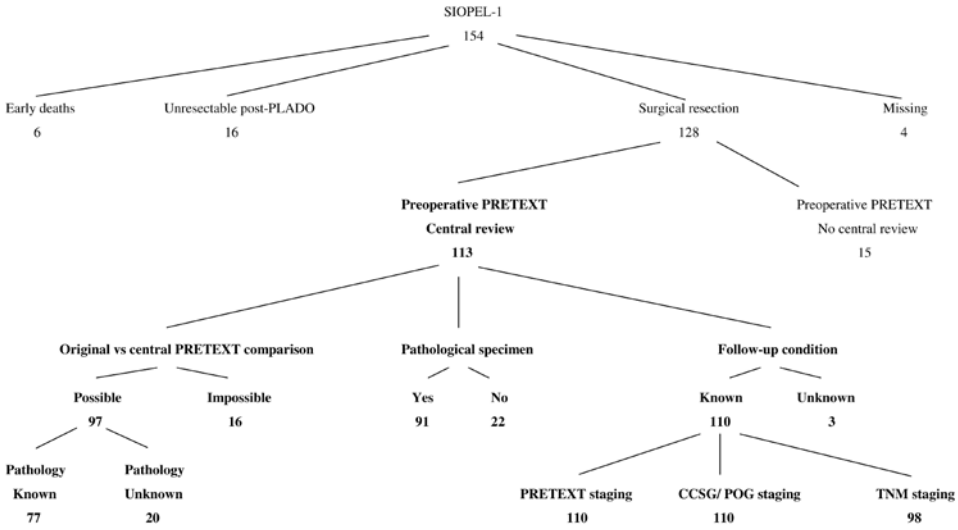
Characteristic	No.	%
Sex		
Male	70	62
Female	43	38
Age (months)		
Median	17	
Range	0-155	
Serum α-fetoprotein (ng/ml)		
Median	172,71[4]	
Range	2-40x10[6]	
Platelet count >500x109/L	66	58
Solitary tumor	89	79
Pulmonary metastases (chest x-ray or lung CT scan)	20	18
Follow-up time (months)		
Median	60	
Range	0-99	
Lost to follow-up	3	–

HB: hepatoblastoma; SIOPEL: Liver Study Group of the International Society of Pediatric Oncology; PRETEXT: pretreatment extent of disease; CT: computed tomography.

The accuracy of PRETEXT: staging before surgery vs. pathological specimen

In 91 patients (81%), exact tumor location in the liver could be traced from the pathology report (ie, '*the gold standard*'; FIGURE 2) and could, thus, be compared with the preoperative PRETEXT staging system after central review.

FIGURE 2 *Flow diagram of the 154 patients who were younger than 16 years with HB and who were registered onto the Liver Tumor Study Group of the International Society of Pediatric Oncology (SIOP) SIOPEL-1 study between January 1990 and February 1994*



PLADO: cisplatin and doxorubicin; PRETEXT: pretreatment extent of disease; CCSG/POG: Children's Cancer Study Group/ Pediatric Oncology Group; TNM: tumor-node-metastasis.

In 22 patients, the pathology report was not available. Fifty one percent of the patients (46 of 91 patients) were staged correctly (ie, tumor found in the sectors predicted by the PRETEXT staging system). In 37% of the patients (34 of 91 patients), the PRETEXT staging was too high (*overstaged*), compared with the exact tumor localization, whereas in 12% of patients (11 of 91 patients), staging was too low (*understaged*). A positive resection margin was found in 4 of these 11 patients, the other 7 children underwent a complete surgical resection. Of the 4 patients with positive resection margins, none developed a local recurrence, which demonstrated the tumor negative status of the unresected liver segments in all patients.

The cross tabulation of the preoperative (centrally reviewed) and postoperative PRETEXT staging according to the pathology report (ie, '*the gold standard*') is shown in TABLE 3. The weighted κ value was 0.44 (95% CI, 0.26 to 0.62).

Table 3 *The preoperative (centrally reviewed) and postoperative PRETEXT staging of 91 patients who entered the SIOPEL-1 study*

		Postoperative PRETEXT (pathology report)				
		Group I	Group II	Group III	Group IV	Total (%)
Preoperative PRETEXT (central review)	Group I	7	3	0	0	10 (11)
	Group II	12	30	8	0	50 (55)
	Group III	3	18	7	0	28 (31)
	Group IV	0	0	1	2	3 (3)
	Total (%)	22 (24)	51 (56)	16 (18)	2 (2)	91 (100)

NOTE. The pathology report was not available in 22 patients. The weighted κ value is 0.44 (95% CI, 0.26 to 0.62).

PRETEXT, pretreatment extent of disease; SIOPEL, Liver Tumor Study Group of the International Society of Pediatric Oncology.

Interobserver agreement: original vs. centrally staged preoperative PRETEXT

In 97 patients (86%), original PRETEXT preoperative staging could be compared with the centrally obtained staging. In 16 patients, one or both PRETEXT stagings were missing (Figure 2). There was an interobserver agreement in 79% of the patients (77 of 97 patients; Table 4). The weighted κ calculated by comparing the original and central PRETEXT staging preoperatively of the 97 patients, was 0.76 (95% CI, 0.64 to 0.88); on the basis of this 95% CI, we have a 95% certainty that the κ lies between 0.64 and 0.88 (ie, good agreement). For the 77 patients (68%) in whom the pathological data were also available, the weighted κ was 0.71 (95% CI, 0.56 to 0.86).

Prognosis according to the different staging systems

Survival analysis according to the different staging systems could be performed in 110 patients (97%). Follow-up data were missing for 3 patients. TNM-based staging could only be performed in 98 patients (87%) because of missing data. The results according to the different staging systems are listed in Table 5.

Table 4 *The preoperative original (ie, the staging according to the local center) and centrally reviewed PRETEXT staging of 97 patients who entered the SIOPEL I study*

		Preoperative PRETEXT (original staging)				
		Group I	Group II	Group III	Group IV	Total (%)
Preoperative PRETEXT (central review)	Group I	6	6	0	0	12 (12)
	Group II	2	47	7	0	56 (58)
	Group III	1	3	20	1	25 (26)
	Group IV	0	0	0	4	4 (4)
	Total (%)	9 (9)	56 (58)	27 (28)	5 (5)	97 (100)

NOTE. In 16 patients, data was missing. The weighted κ value was 0.76 (95% CI, 0.64 to 0.88).

PRETEXT, pretreatment extent of disease; SIOPEL, Liver Tumor Study Group of the International Society of Pediatric Oncology.

Table 5 *PRETEXT grouping, CCSG/POG staging and TNM staging of the 110 patients with HB who were treated with surgical resection in the SIOPEL-1 study and were centrally reviewed*[a]

Staging	no.	%	no. died
Preoperative PRETEXT			
Group I	13	12	0
Group II	63	57	3
Group III	29	26	2
Group IV	5	5	3
Total	110	100	–
CCSG/POG staging			
Stage I	77	70	5
Stage II	8	7	1
Stage III	6	6	1
Stage IV	19	17	1
Total	110	100	–
TNM staging			
Stage I	0	0	–
Stage II	63	57	3
Stage III	7	6	3
Stage IV	28	26	2
Missing	12	11	–
Total	110	100	–

PRETEXT: pretreatment extent of disease; CCSG/POG: Children's Cancer Study Group/Pediatric Oncology Group; TNM: tumor-node-metastasis; HB: hepatoblastoma.

[a] See also Figure 2

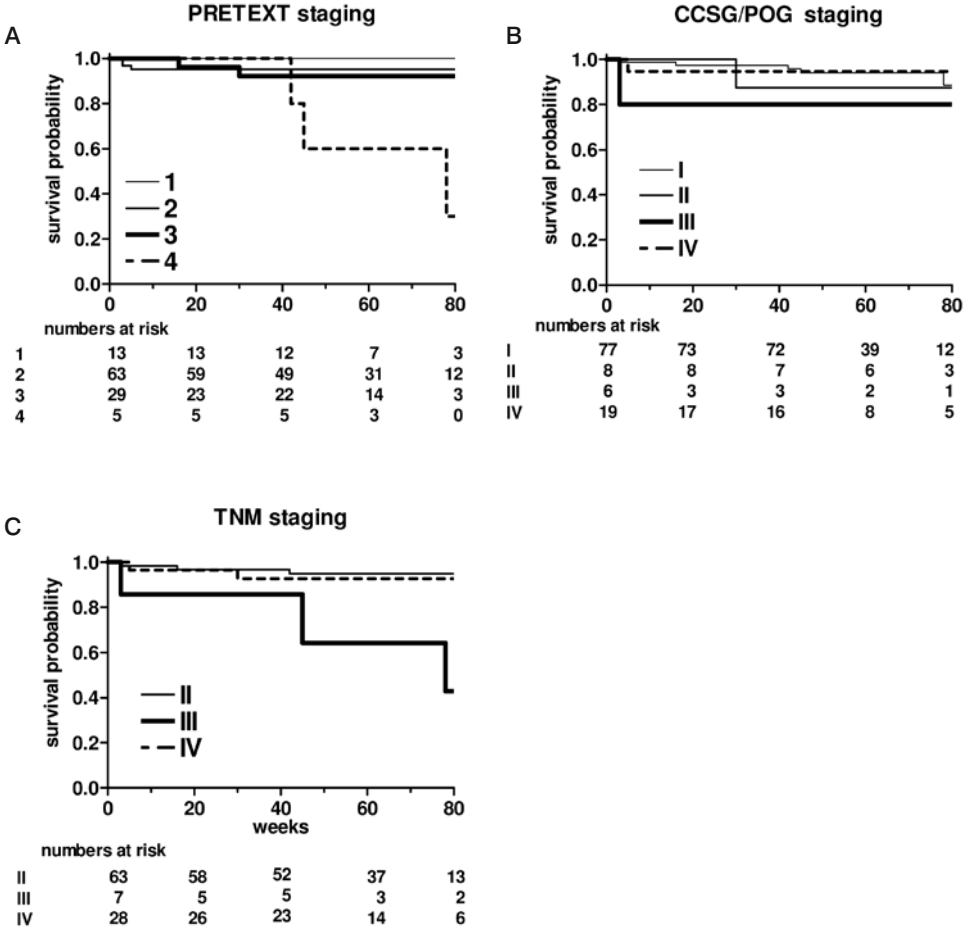
The 5-year overall survival rates according to the different preoperative PRETEXT groups after central review were 100% for group I, 95% for group II, 93% for group III, and 40% for group IV (Figure 3A). This system revealed a decreasing trend in overall survival related to the different subgroups that seemed to be highly significant ($P = 0.0006$).

The 5-year overall survival according to the system used by CCSG/POG-based staging system is presented in Figure 3B. Patients with metastases (stage IV), who had complete surgical resection of their primary tumor (a select group of patients who underwent the exact SIOPEL-1 protocol and who were, therefore included in this analysis), had the same survival rate (95%) as those patients with complete resection without metastases (stage I; 94%), microscopic residual disease (stage II; 88%), or macroscopic residual disease (stage III; 83%). These differences were not significant ($P = 0.516$). Note that there was a difference between the absolute figures of the CCSG/POG-based staging and true CCSG/POG staging; in the original group of 154 patients, 31 patients who entered onto the trial with lung metastases (CCSG/POG stage IV), who showed a 5-year event-free survival rate of 57% and 5-year overall survival rate of 28%, respectively[19]. Finally, the 98 patients who where staged according to the TNM-based staging system (Figure 3C) showed a 5-year overall survival of 95% for stage II patients (stage I did not occur), 57% for stage III patients, and 93% for stage IV patients. Patients with a stage IV tumor who underwent the exact SIOPEL-1 protocol and, therefore, included in this analysis were a select group of patients. The TNM-based staging system seemed to be highly significant in relation to overall survival as well ($P = 0.0021$).

Cox proportional hazards ratios

For each of the 3 staging systems, a Cox proportional model was obtained, which entered the staging levels as independent variables considering the highest level as reference category. AIC was used for comparison of the three models. The best statistical fit was obtained with the PRETEXT staging system, which revealed the lowest AIC score (67.4), followed by the TNM-based staging system (67.9) and the CCSG/POG-based staging system (75.3). The higher AIC score of the CCSG/POG-based staging system indicates the weakest statistical fit.

FIGURE 3 **A.** *The 5-year overall survival according to the different preoperative retreatment extent of disease (PRETEXT) groups after central review (*P = *0.0006, logrank test).* **B.** *The 5-year overall survival according to the system used by the Children's Cancer Study Group/Pediatric Oncology Group (CCSG/POG;* P = *0.516, logrank test).* **C.** *The 5-year overall survival according to the tumor-node-metastasis (TNM) system for (adult) liver carcinomas (*P = *0.0021, logrank test)*



Discussion

In the last decade large international, study protocols for the treatment of children with HB have been developed in the United States, Germany, and Japan and by the SIOPEL group[3-5,20-22]. Currently, overall survival rates lie in the range of 75 to 80%, and event-free survival rates range from 57 to 69%[5,7,9,22]. In this respect, the various protocols or treatment strategies do not show large differences in outcome. The different study groups used several staging systems, of which, all were reported to be significant in respect to prognostic relevance. The drawback to the use of different staging systems is that patients and, thus, study results are difficult to compare. Almost all groups use postoperative staging. The CCSG/POG study groups and the German group used the same postoperative system, which the German group compared with the prognostic relevance of the adult liver carcinoma TNM-system of the International Union Against Cancer[13,23], and a Japanese study group proposed the postoperative Japanese TNM-system[24]. The German group advised the use of the TNM system for comparison of the treatment results in HB but stated that a disadvantage of the TNM staging systems is that they are based on postoperative pathologic findings and, therefore, can only be applied to patients who underwent surgery. Therefore, the advantages of the preoperative imaging-based staging system developed by the SIOPEL-1 study group are that it can be applied to all patients, it can be used to monitor the effect of preoperative chemotherapy, and it can assess the resectability of the tumor and the required type of resection *before* surgery.

To assess the accuracy of the PRETEXT system, the preoperative PRETEXT staging was compared with the pathology report of the postoperative resection specimen (ie, the '*gold standard*'). Therefore this could only be applied to patients who underwent surgery, which is a selected subgroup of all HB patients. Our data showed that only 46 (51%) of 91 tumors were correctly staged, with a tendency to *overstage* the tumor (37%). For example, tumors were staged as group IV (ingrowth), whereas, in fact, they should have been staged as group III (compression). This phenomenon may be explained by the difficulty, if not impossibility, of distinguishing parenchymal compression of a tumor-free liver sector from tumor ingrowth into that sector. The weighted κ value of 0.44 supports this assumption because it means that the accuracy of PRETEXT is moderate. Hopefully, future improvement of imaging quality and obligatory central review may improve this discrepancy, maybe even by using other imaging techniques, like magnetic resonance imaging. However, this assumption has to be studied prospectively.

However, the interobserver agreement of staging tumors according to the PRETEXT system is good as shown by the weighted κ value of 0.76. This means that the system is reproducible, and one might assume that the system can easily be applied by different clinicians and that a relative uniformity of tumor staging exists. Although, one has to keep in mind that 63% of all patients (97 of 154 patients) who were eligible on the SIOPEL-1 study had their local PRETEXT staging compared with central staging, and 50% of all patients (77 of 154 patients) who were eligible on the SIOPEL-1 study were available to compare pathology with pretreatment staging (see flow chart in Figure 2).

Similarly, only 64% (98 of 154 patients) to 71% of the patients (110 of 154 patients) were available for comparing the three different staging systems in use for HB (Figure 2). Still, our data show that the predictive value in relation to survival of the PRETEXT system is at least as good as the well-known TNM-based system. Both systems had a highly significant predictive value in relation to survival in the SIOPEL-1 study. In contrast, in this select group of patients, the CCSG/POG-based system seemed to be not significantly related to survival, probably because most patients had stage I disease. This finding was also confirmed by the statistical fit of the three staging systems in the Cox proportional hazards models, which showed a superiority for the PRETEXT system.

In conclusion, the results of the present data show that the accuracy of the PRETEXT system is moderate when the pre- and postoperative stages are being compared, probably as a result of the difficulty to distinguish parenchymal compression from true parenchymal ingrowth of the tumor; there was a tendency to *overstage* the patients; and the PRETEXT system demonstrated a good interobserver agreement, which means that this staging system is reproducible. The predictive value for survival of PRETEXT and of the TNM-based system was highly significant in contrast to the predictive value of the CCSG/POG-based system. However, the PRETEXT system has an advantage because it offers the opportunity to monitor the effect of the neoadjuvant therapy used *before* surgery. Further research is necessary to evaluate the predictive value of PRETEXT in patients who do not receive surgical resection to evaluate the predictive value of this PRETEXT system and its use in monitoring the effects of preoperative chemotherapy, not only in patients who receive surgical resection, but in all patients. We recommend that all patients with HB included in the trials from the different study groups be staged both by their own preferred staging system as well as according to the PRETEXT system. This offers the opportunity to monitor preoperative treatment and to compare the results from the various trials in a more accurate way.

Acknowledgement

We would like to emphasize that this study could only be conducted with the participation of the centers summarized in Appendix I.

References

1. Mann JR, Kasthuri N, Raafat F, et al: Malignant hepatic tumours in children: incidence, clinical features and aetiology. *Paediatr Perinat Epidemiol* 1990; 4:276-89.
2. Ortega JA, Krailo MD, Haas JE, et al: Effective treatment of unresectable or metastatic hepatoblastoma with cisplatin and continuous infusion doxorubicin chemotherapy: a report from the Childrens Cancer Study Group. *J Clin Oncol* 1991; 9:2167-76.
3. von Schweinitz D, Byrd DJ, Hecker H, et al: Efficiency and toxicity of ifosfamide, cisplatin and doxorubicin in the treatment of childhood hepatoblastoma. Study Committee of the Cooperative Paediatric Liver Tumour Study HB89 of the German Society for Paediatric Oncology and Haematology. *Eur J Cancer* 1997; 33:1243-9.
4. Douglass EC, Reynolds M, Finegold M, et al: Cisplatin, vincristine, and fluorouracil therapy for hepatoblastoma: a Pediatric Oncology Group study. *J Clin Oncol* 1993; 11:96-9.
5. Brown J, Perilongo G, Shafford E, et al: Pretreatment prognostic factors for children with hepatoblastoma – results from the International Society of Paediatric Oncology (SIOP) study SIOPEL 1. *Eur J Cancer* 2000; 36:1418-25.
6. Pritchard J, Brown J, Shafford E, et al: Cisplatin, doxorubicin and delayed surgery for childhood hepatoblastoma a successful approach. Results of the first prospective study of the international society of pediatric oncology (SIOP) - SIOPEL 1. *J Clin Oncol* 2000; 18: 3819-28.
7. Schnater JM, Aronson DC, Plaschkes J, et al: Surgical view of the treatment of patients with hepatoblastoma. Results from the first prospective trial of the International Society of Paediatric Oncology Liver Tumor Study group (SIOPEL-1). *Cancer* 2002; 94:1111-20.
8. Plaschkes J, Vos A, Czauderna P, et al: Into the year 2000 with the SIOPEL III - cisplatin (CDDP) alone or in combination for standard and high risk hepatoblastoma, the SIOP XXX meeting. *Med Pediatr Oncol* 1998; 31:240
9. Von Schweinitz D: Identification of risk groups in hepatoblastoma – another step in optimising therapy. *Eur J Cancer* 2000; 36:1343-6.
10. MacKinlay GA, Pritchard J: A common language for childhood liver tumours. *Pediatr Surg Int* 1992; 7:325-6.
11. Vos A: Primary liver tumours in children. *Eur J Surg Oncol* 1995; 21:101-5.
12. Hermanek P, Sobin J: UICC: TNM classification of malignant tumours (ed 4). Berlin, Springer, 1992
13. von Schweinitz D, Hecker H, Schmidt-von-Arndt G, et al: Prognostic factors and staging systems in childhood hepatoblastoma. *Int J Cancer* 1997; 74:593-9.
14. Couinaud C: Lobes et segments hepatiques. *Presse Med* 1954; 62:709-12.
15. Sobin LH, Wittekind C: UICC: TNM Classification of malignant tumours (ed 5). New York, J Wiley & Sons, 1997
16. Akaike H: A new look at the statistical model identification. *IEEE Trans Autom Contr* 1974; 19:716-23.
17. Kaplan EL, Meier P: non-parametric estimation from incomplete observations. *J Amer Statist Ass* 1958; 53:457-81.
18. Kalbfleisch JD, Prentice RL: The statistical analysis of failure time data. New York, J Wiley, 1980
19. Perilongo G, Brown J, Shafford E, et al: Hepatoblastoma presenting with lung metastases. *Cancer* 2000; 89:1845-53.
20. Evans AE, Land VJ, Newton WA, et al: Combination chemotherapy (vincristine, adriamycin, cyclophosphamide, and 5-fluorouracil) in the treatment of children with malignant hepatoma. *Cancer* 1982; 50:821-6.
21. Sasaki F, Ohmuna N, Iwafuchi M: Outcome of hepatoblastoma in children treated with the JPLT protocol. *Med Pediatr Oncol* 1998; 32:308.
22. Ortega JA, Douglass EC, Feusner JH, et al: Randomized comparison of cisplatin/vincristine/fluorouracil and cisplatin/continuous infusion doxorubicin for treatment of pediatric hepatoblastoma: A report from the Children's Cancer Group and the Pediatric Oncology Group. *J Clin Oncol* 2000; 18:2665-75.
23. von Schweinitz D, Burger D, Mildenberger H: Is laparatomy the first step in treatment of childhood liver tumors? – The experience from the German Cooperative Pediatric Liver Tumor Study HB-89. *Eur J Pediatr Surg* 1994; 4:82-6.
24. Morita K, Okabe I, Uchino J, et al: The proposed Japanese TNM classification of primary liver carcinoma in infants and children. *Jpn J Clin Oncol* 1983; 13:361-9.

'From the clinical aspects back to the laboratory!

Gaining insight in the phenotypical aspects of hepatoblastoma may help to understand the tumorigenetic process and may show us some probable prognostic factors'

Chapter 5

Architectural markers in hepatoblastoma

A morphological study

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Abstract

Background: The biology of hepatoblastoma (HB) is still incompletely understood. The present study investigated the phenotype of the nodules, the smallest structural feature of hepatoblastoma.

Methods: Six biopsies and 18 surgical resection specimens of 19 children (15 of the epithelial type and 4 of the mixed type) were studied immunohistochemically for expression of carbamoylphosphate synthetase (CPS), glutamine synthetase (GS), α-fetoprotein, and cytokeratins 7 and 19 (CK7, CK19).

Results: Infant liver exhibits a near-adult expression pattern of proteins, except that CPS is still homogeneously expressed. Furthermore, more epithelial cells express CK7 than CK19, suggesting that CK7 identifies Hering's duct. Most HBs appeared to grow slowly or invasively. CPS and GS expression was remarkably homogeneous in the smaller nodules. Expression of CPS was found in >80% of HBs, but absent in 3 of 4 mixed-type HBs. GS expression in larger nodules was confined to the nodular periphery. No correlation between GS and β-catenin expression was observed, even though GS is an established downstream target of β-catenin. CK7- and CK19-positive cells surrounded GS-positive tumor nodules, usually in association with expansive growth. The finding of a phenotypically different nodule within a larger, itself homogeneous nodule indicated that "founder" effects were an important source of phenotypic variation among nodules. The presence of afferent vessels in the absence of bile ducts ("unpaired" vessels) showed that nodular vascularization differs from that of lobules. The colocalization of GS and CK7/CK19 in the nodular periphery appears to reflect the independent effects of epithelio-mesenchymal interactions (CK expression) and vascular gradients in metabolites and signaling factors (GS), and suggests that such nodules are still fully responsive to environmental stimuli.

Conclusion: Characteristics in the growth pattern (expansive vs. infiltrative), the size of phenotypically homogeneous nodules (reflecting the rate of dedifferentiation), the vascularization pattern (with perfusion by an "unpaired" central artery) and the zonation of gene expression in the nodules are proposed as new prognostic factors that are based on the architectural and phenotypic properties of the tumor cells within a hepatoblastoma.

Introduction

Hepatoblastoma (HB) and hepatocellular carcinoma (HCC) are the most common primary liver tumors in children[1]. Currently, the 5-years overall survival rate is 75% and 30-40%, respectively[2-5]. Currently, alpha-fetoprotein (αFP) is used as a tumor marker because 90-95% of the patients with a HB and ~40-90% of patients with HCC show elevated levels of serum αFP[2,6]. Since the prognosis is much better for HB, the attention focused to tailored therapy, based on the identification and evaluation of risk groups and risk factors[3]. These studies revealed that children with an extrahepatic tumor extension, multifocality, vascular invasion, low αFP, and distant metastases have a poorer prognosis[7-9].

Many tumors, including HB, are characterized by genotypic and phenotypic heterogeneity[10-12]. The prognostic role of the tumor cell type and phenotypic heterogeneity within the intrahepatic tumor is less well established. Most pathologists distinguish hepatoblastomas of the *epithelial* type, which contains hepatoblastoma cells with an embryonal or a fetal hepatocellular phenotype, or a mixture of the two, from the *mixed* type that contains mesenchymal tissue in addition to the epithelial elements[3]. The putatively more favorable prognosis of the pure fetal type and the less favorable prognosis of the anaplastic (also known as small cell undifferentiated) type demonstrate the uncertainties of the prognostic relevance of these histological characteristics. To address this issue, several studies have proposed new prognostic factors that are based on the phenotypic properties of the tumor cells within a hepatoblastoma[7,13-18]. Other studies have identified stem cell markers[19-21], tumor marker[22-25], and prognostic factors in HB[7,8,12,14,26], but the phenotypic homogeneity (i.e. the degree of dedifferentiation), the presence of connective tissue and vessels (i.e. the architecture as reflection for autonomic growth or production of growth factors), and the response of normal tissue surrounding the tumor have not yet been studied as potential prognostic markers. In this study, we describe the expression pattern of a number of marker proteins in 19 HBs.

In adult liver, *carbamoylphosphate synthetase* (CPS), the first and rate-determining enzyme of the urea cycle, is normally expressed in the hepatocytes surrounding the portal veins (the 'periportal area'; Figure 1)[27,28]. CPS becomes expressed in human hepatocytes soon after these cells differentiate from the embryonic foregut and also behaves as an early hepatocyte marker in experimental models[29-33].

Cytokeratin 19 (CK19), also an intermediate filament, is a marker for some epithelial cells, such as bile duct cells (Figure 1) but, more importantly, is also a strongly expressed marker for liver stem cells[19,21,34-36]. Its up-regulation has already been described in HB and HCC[37-39].

Another marker of biliary differentiation is *cytokeratin 7* (CK7). This cytokeratin is expressed in cells of morphology and immunophenotype intermediate between hepatocytes and cholangiocytes[40]. These 'intermediate cells', also known as 'progenitor cells', are probably the small epithelial cells closely related to the putative bipotent hepatic stem cell which play a role in HB[19,34]. Like CK19, the overexpression of CK7 is also described in HB[19,38,39].

The development of stabilizing mutations in the *β-catenin* gene and nuclear translocation of the corresponding protein occurs in many gastrointestinal and hepatic carcinomas[41-47]. Since the activation and nuclear translocation of β-catenin is not easily demonstrable, we have also studied the expression of *glutamine synthetase* (GS), a well-known downstream target of β-catenin signaling in the liver[48]. In normal human liver, GS is expressed in a small rim of cells around the central veins (the 'pericentral area'; Figure 1). Up-regulation of GS expression in HCC has been described[48-52], but as far as we know, its expression in HB in vivo is limited to subcutaneous explants in immune-deficient mice[53].

We have used the expression of the aforementioned marker proteins to explore whether or not the tumor cells still express a hepatocellular phenotype, contain stem cells, or suffer from β-catenin activation. Furthermore, we investigated whether or not the tumors grew by expansion or also infiltrated the surrounding liver tissue. Finally, we investigated the degree of phenotypic heterogeneity within and between tumor nodules.

Patients and methods

Six biopsies (2 open cut) and 18 surgical resection specimens of 19 children with HB were studied (Table 1). The children ranged in age from 4 months to 3 years. Fifteen samples were morphologically staged by the pathologist as epithelial type and 4 as mixed type HBs. The histological material was supplied by 4 different hospitals via their departments of pathology. Part of all liver specimens was routinely processed for diagnostic evaluation, the remainder was snap-frozen in liquid nitrogen and stored at −70°C. The diagnosis of HB was made in the local department of pathology, based on standard diagnostic criteria.

Table 1 *The demographic and histological data of the 19 children with hepatoblastoma*

Case #	Sex[a]	Age (mo)	Histology	Chemotherapy	Specimen[b]	Outcome[c]
1	F	4	HB epithelial	+	Bx & R	NED
2	F	4	HB mixed	+	Bx & R	DOD
3	F	10	HB epithelial	+	Bx & R	NED
4	M	10	HB epithelial	+	R	NED
7	F	12	HB mixed	+	R	DOD
8	F	12	HB epithelial	+	R	ND
9	M	14	HB mixed	+	R	NED
10	F	16	HB epithelial	+	R	NED
11	M	17	HB epithelial	+	R	NED
12	F	21	HB epithelial	+	Bx & R	NED
14	F	36	HB epithelial	+	R	NED
15	ND	ND	HB epithelial	+	R	ND
5	F	11	HB mixed	–	Bx & R	DOD
13	M	24	HB epithelial	–	R	DOD
6	M	12	HB epithelial	ND	Bx	ND
16	ND	ND	HB epithelial	ND	R	ND
17	M	ND	HB epithelial	ND	R	ND
18	F	ND	HB epithelial	ND	R	ND
19	F	ND	HB epithelial	ND	R	ND

[a] F = female, M = Male. [b] Bx = biopsy, R= resection specimen. [c] DOD = died of disease, NED = no evidence of disease, mo = months, HB = hepatoblastoma. ND = no data.

Twelve liver tumor samples were pretreated with chemotherapy (2 died), 2 were not pretreated (both died), and in 5 data about the pretreatment were not available (and the outcome not known).

Immunohistochemistry was preferably performed on methanol/acetone/H_2O or formaldehyde-fixed paraffin-embedded serially cut sections. Slides were pretreated with 0.3% hydrogen peroxide/70% ethanol solution for 1 hour at room temperature to inactivate endogenous peroxidase activity. An indirect unconjugated peroxidase-antiperoxidase staining procedure was used. The sections were incubated with the primary antibodies overnight in a humidity chamber at room temperature. Monoclonal β-catenin, αFP, GS, (Transduction Laboratories, Lexington KY, USA), CK7 (Progen Biotechnik GmbH, Heidelberg, Germany) and CK19 (clone LP2K, Amersham, Buckinghamshire, UK), and polyclonal CPS[54] dilutions were used. All antibodies were diluted in 300mM Na-acetate. This was followed by incubation with rabbit-anti-mouse serum (monoclonals) and goat-anti-rabbit serum (polyclonal), and finally by rabbit-peroxidase-anti-

Table 2 *The expression of the different architectural markers in 19 children with hepatoblastoma*

Case #	Growth pattern	CPS			GS			CK7		CK19	
		tumor[a]	*heterogeneity expression*	*nodular*	*tumor*[a]	*heterogeneity expression*	*nodular*	*tumor*	*surrounding*	*tumor*	*surrounding*
1	expansive	+	homogeneous	–	+	homogeneous	–	–	+	–	–
2	infiltrative	–			–			ND	ND	ND	ND
3	expansive	++	homogeneous	–	++	homogeneous	–	–	+	+	+
4	expansive	++	heterogeneous	+	+	homogeneous	–	–	+	–	+
7	expansive	++	heterogeneous	+	++	heterogeneous	+	–	+	+	+
8	infiltrative	++	heterogeneous	+	++	heterogeneous	+	ND	ND	–	–
9	infiltrative	–			–			ND	ND	ND	ND
10	infiltrative	+	homogeneous	–	+++	homogeneous	–	–	ND	–	ND
11	infiltrative	++	heterogeneous	+	++	homogeneous	–	ND	ND	–	+
12	expansive	++	homogeneous	+	+++	homogeneous	–	–	+	–	+
14	expansive	++	homogeneous	+	++	homogeneous	+	ND	ND	ND	ND
15	infiltrative	++	homogeneous	–	+	homogeneous	–	ND	ND	–	ND
5	infiltrative	–			–			–	+	+	+
13	expansive	++	heterogeneous	+	++	heterogeneous	+	ND	ND	ND	ND
6	expansive	++	heterogeneous	–	+	heterogeneous	–	ND	ND	–	+
16	infiltrative	++	heterogeneous	+	–			ND	ND	–	+
17	infiltrative	+	homogeneous	–	+++	homogeneous	–	ND	ND	–	–
18	infiltrative	++	heterogeneous	+	++	heterogeneous	+	ND	ND	ND	ND
19	infiltrative	+	homogeneous	–	+	homogeneous	–	ND	ND	–	–

[a] – = no expression. + = weak expression. ++ = normal expression. +++ = strong expression.
CPS= carbamoyl phosphate synthetase. GS= glutamine synthetase. CK7= cytokeratin7. CK19= cytokeratin19. ND= no data.

peroxidase complex for 1.5 hour. Preimmune serum was used as a negative control. When available, normal liver tissue of the same patient was used as a positive control. All slides were stained with diaminobenzidine solution (DAB Tablets, Amresco, Cleveland, Ohio). The morphological aspects of the tumor were studies in standard hematoxylin/eosin stained sections.

The tumor samples were scored for: (1) the expression of GS, CPS, CK7, CK19 and, in a few cases, αFP; (2) the degree to which these markers were expressed (homogeneous, heterogeneous or apparently lost); (3) the relation of the observed expression patterns to the presence of connective tissue and blood vessels; (4) the growth pattern (infiltrating or expansive) of the tumor (TABLE 2).

Because the patient's condition during the last follow-up could only be traced in 12 out of 19 patients with HB (63%), we did not attempt to derive a clear prognostic value with respect to survival for the different growth patterns or, for that matter, for the different expression patterns of the markers.

Results

Normal expression pattern

Figure 1 shows that the expression pattern of CPS, GS, CK7 and CK19 in the liver of an 16-months old child is reminiscent to that seen in adults (cf. ref [49]). However, CPS is still expressed in all hepatocytes, even though a slight gradient in staining intensity is seen that declines from the portal to the central veins. As in the adult, GS is only expressed in the hepatocytes in the immediate vicinity of the central veins, while the hepatocytes surrounding the larger, sublobar veins no longer express GS (cf. ref [55]). CK7 and CK19 expression is confined to the bile ducts, but more epithelial cells stain positive for CK7 than for CK19. The cells expressing CK7, but not CK19 represent those lining the duct of Hering[19,34].

FIGURE 1 (*see colour pages 139*) *Expression pattern of carbamoyl phosphate synthetase (CPS), glutamine synthetase (GS), and cytokeratins (CK) 7 and 19 in normal human liver tissue of a 11 month-old female. Panels A-D are serial sections. CPS (A) is expressed homogeneously across the liver lobule, GS (B) is confined to the hepatocytes surrounding central veins (CV), while CK7 (C) and CK19 (D) are expressed in bile-ductular structures bordering the portal tracts. Note that more cells express CK7 than CK19 (for details see panels E and F).*

Growth pattern of the tumors

We mostly observed a nodular growth pattern of HB tumors. In 11 out of 19 specimens (~60%), the hepatic tissue surrounding these HB nodules did not show signs of compression, suggesting that the tumors grew very slowly or invasively. In these cases, higher magnifications showed that the cells at the periphery of the tumor indeed appeared to grow invasively (FIGURE 2). FIGURE 3

shows on the other hand a rare small nodule with the normal hepatocytes surrounding it, stretched in a way that suggests rapid expansion of the node.

Figure 2 (*see colour pages 140*) *Hepatoblastoma nodule in the liver of a 10 months-old female showing infiltrative growth. Panels A-D are serial sections. The nodule is carbamoyl phosphate synthetase positive (A), nearly glutamine synthetase- (B) and keratin-negative (D), while many of the infiltrating strands are cytokeratin-positive (C).*

Figure 3 (*see colour pages 140*) *Hepatoblastoma nodule in the liver of a 11 months-old female inducing a stretched appearance in the surrounding hepatocytes (panel A and B, stained for the presence of carbamoylphosphate synthetase (CPS) and glutamine synthetase (GS), respectively). Panels A-D are serial sections. The nodule itself does not express CPS or GS, has lost its epithelial character, and is special in that it expresses cytokeratin19 (D), but not cytokeratin 7 (C).*

Sometimes, a fibrous (pseudo)capsule that is suggestive of expansive growth was infrequently found (e.g. Figure 4). Nevertheless, many tumors contained thick, fibrotic sheaths surrounding the nodules. Hepatocytes that became trapped between nodules gradually accumulated CK7 and, to a lesser extent, CK19 (Figure 5), suggesting transdifferentiation into bile ductular structures. Eventually, the hepatocytes lost CPS expression and were only positive for bile duct markers (not shown). Entrapment of hepatocytes and the assumption of a bile-ductular phenotypes was also found in tumors that had not been treated with chemotherapy (cases # 5 and 13).

Figure 4 (*see colour pages 141*) *Hepatoblastoma nodule in the liver of a 24 months-old male showing expansive growth. Panels A-C are serial sections. The gene expression pattern in the nodule is heterogeneous, with almost all epithelial cells expressing carbamoyl phosphate synthetase (A) and the peripheral epithelial cells glutamine synthetase (B), whereas cytokeratin-7 (C) is hardly expressed. Note extensive erythropoiesis.*

Figure 5 (*see colour pages 141*) *Liver tissue adjacent to a hepatoblastoma nodule. Panels A-D are serial sections. All hepatocytes still express carbamoyl phosphate synthetase (A), but glutamine synthetase expression is nearly extinguished (B). In addition to numerous bile-ductular structures, cytokeratin7 weakly stains the hepatocytes (C), whereas cytokeratin19 staining is confined to the bile-ductular structures (D).*

We were unable to demonstrate tumor-cell proliferation with Ki67 staining of the nodules (intestinal samples processed concurrently were clearly positive; data not shown). Also in agreement with a relatively slow growth pattern was the presence of well-developed vascular trees and the absence of necrotic areas inside the larger nodules (Figures 6 and 7).

Figure 6 (*see colour pages 142*) *Hepatoblastoma nodule in the liver of a 21 months-old female. Panels A-D are serial sections. The tumor barely distinguishes itself from the surrounding liver tissue in sections stained with haematoxylin and azophloxin (A), cytokeratin7 ((CK7) (B), and carbamoyl phosphate synthetase (C). However, staining for the presence of glutamine synthetase (D) clearly delineates the nodule. CK7 stains the cells lining the tumor nodule (C).*

Figure 7 (*see colour pages 143*) *Large hepatoblastoma nodule from the same patient shown in Figure 6. Panels A, B, E and F are serial sections. The tumor nodule expresses carbamoyl phosphate synthetase ((CPS); A)), glutamine synthetase ((GS); B), but neither cytokeratin7 (E) nor cytokeratin19 (F). The magnifications (C and D) show that the vascular tree was present in small tracts and no longer accompanied by bile ducts ("unpaired" arteries). They further show a clear zonation of expression for CPS (C) and to a much lesser extent GS (D).*

Gene expression patterns of tumor nodules

Carbamoylphosphate synthetase (CPS)

The expression of the hepatocyte markers CPS, GS, and αFP was remarkably homogeneous in the smaller nodules. Carbamoylphosphate synthetase expression was found in 16 out of the 19 tumor samples (>80%). Most tumors did express CPS at a level that was indistinguishable from the surrounding "healthy" tissue (Figures 6 and 7). However, some nodules expressed CPS at a lower level than that present in hepatocytes (Figure 8), or might have lost CPS expression (Figure 9). Figures 8 and 9 are from the same liver, suggesting that nodules can lose the capacity to express a gene. In this respect, the staining pattern of Figure 10 is even more intriguing as it shows the appearance of a well-demarcated nodule that hardly expressed either CPS or GS within a nodule that is CPS- and strongly GS-positive. The cellular appearance of this CPS-, GS-negative nodule also differs from the parent nodule (Figure 10D), suggesting that the loss of CPS and GS expression is secondary and reflects a dedifferentiation event. The tumors in 3 out of 4 cases with a hepatoblastoma of the mixed type had entirely lost their CPS expression (Figure 3). A closer look at the histology of the tumor cells showed that they had also lost their typical epithelial character (Figure 3). Loss of CPS expression could not be attributed to preoperative chemotherapy, because case # 5 was not pretreated.

Figure 8 (*see colour pages 142*) *Hepatoblastoma nodules in the liver of a male patient (age unknown; case # 17). Panels A, C, and D are serial sections, while panel B is a magnification of panel A. Haematoxylin & azophloxin stained sections (A and B). The nodule stains only weakly for carbamoyl phosphate synthetase (C), but is strongly glutamine synthetase-positive (D).*

Figure 9 (*see colour pages 144*) *Hepatoblastoma nodule in the liver of the same patient shown in Figure 8. Panels A-D are serial sections. The nodule shown has lost expression of carbamoyl phosphate synthetase (A), but is strongly positive for glutamine synthetase (B) and weakly for α-fetoprotein (C). These panels, in particular D (H&A stained) suggest that this nodule resides in a vessel, in other words, would represent a metastasis.*

Figure 10 (*see colour pages 144*) *Hepatoblastoma in the liver of a 21 months-old female showing a nodule-within-a-nodule. Panels A and B are serial sections, while panel C is a few sections further away. The outer, parent nodule is carbamoyl phosphate synthetase- (A) and glutamine synthetase-positive (B). The nodule within the nodule, on the other hand, does not express either gene. As panel C and D (H&A stained) show, the nodule within the nodule also consists of cells that differ from the parent nodule.*

Alpha-fetoprotein (αFP)

Alpha-fetoprotein could only be detected well in cryostat sections of tumors that were not pre-treated with chemostatic agents (Figures 9 and 11). In some nodules, its presence coincided with that of CPS (Figure 11), whereas in others (Figure 9), it did not.

Figure 11 (*see colour pages 145*) *Hepatoblastoma nodules in the liver of the same patient shown in Figure 10. Panels A-C are serial sections. All nodules behave phenotypically similar and are positive for carbamoyl phosphate synthetase (A), glutamine synthetase (B), and α-fetoprotein (C).*

Glutamine synthetase (GS)

The expression pattern of GS was more diverse than that of CPS. In many nodules, it was strongly and homogeneously expressed (Figures 6,7,8,9,11), whereas in others its expression was extremely weak (Figure 3). Figure 13 shows clear differences in the level of GS expression between adjacent nodules. In this case, CPS expression was also different in these nodules. In the GS-negative nodules, a few cells remained intensely GS-positive. As already stated in the paragraph about CPS expression, Figure 10 shows the appearance of a well-demarcated nodule that hardly expressed either CPS or GS within a nodule that is CPS negative and strongly GS-positive. In larger nodules, in which all cells expressed GS (figure 7), shallow gradients in expression were seen that opposed similarly shallow gradients in CPS expression, with CPS being highest around the feeding vessels and GS away from them. In still other cases, the expression gradient of GS was more prominent, with the highest level of expression being present in the nodular periphery (Figures 4 and 14).

Figure 12 (*see colour pages 145*) *Large hepatoblastoma nodule in the liver of a 12 months-old female. Panels A-C are serial sections. Carbamoyl phosphate synthetase is homogeneously expressed (A), but cytokeratin7 (B) and glutamine synthetase (C) are heterogeneously expressed, both being highest at the periphery of the lobular indentations at the interphase with connective tissue.*

Figure 13 (*see colour pages 146*) *Hepatoblastoma nodules in the liver of 10 months-old male showing marked differences in gene expression. Panels A-C are serial sections. The two upper and outward nodules are carbamoyl phosphate synthetase- ((CPS); A) and glutamine synthetase- ((GS); B) positive, whereas the lower and inner nodules are CPS-positive, but GS-negative. All nodules are cytokeratin7-negative (C).*

Figure 14 (*see colour pages 146*) *Hepatoblastoma nodule in the liver of a 10 months-old female. Panels B-D are serial sections, while panel A is a few sections away. Panel A shows an H&A staining, while panels B-D shows the expression of carbamoyl phosphate synthetase (CPS), glutamine synthetase (GS), and cytokeratin19 (CK19), respectively. Note homogeneous expression of CPS and restriction of expression of GS and CK19 to the periphery of nodule.*

Cytokeratins

The expression patterns of CK7 and CK19 were complex. We observed areas adjacent to the tumor that still showed a near-normal lobular architecture (Figure 15), expressed CPS homogeneously and GS around central veins. However, these lobules were surrounded by a "chicken wire" of CK19-positive cells. Often, CK7- and CK19-positive cells also surrounded clear-cut, GS-positive tumor nodules (Figure 6, 14, 16). This peripheral CK expression was found in 6/7 expansively growing tumors and in 3/6 infiltratively growing tumors ($p = 0.16$, X^2 test). In none of the 7 stained tumors, CK7 was expressed by the tumor cells, whereas in only 3/14 tumors stained, CK19 was expressed by the tumor cells. In 2 of these 3 tumors, CK19 was expressed in the peripheral-most cells (Figure 14), whereas in one case (Figure 3), CK19 expression was homogeneous. In most cases, therefore, expression of both cytokeratins was absent or near-absent (Figures 7, 9, and 13).

Figure 15 (*see colour pages 148*) *Hepatoblastoma nodules in the liver of a 17 months-old male. Panels A-C and D-F are serial sections. Note near homogeneous expression of carbamoyl phosphate synthetase (A and D), confinement of glutamine synthetase to central vein-like vessels (B and E), and numerous cytokeratin19-positive bile ducts surrounding the nodules (C and F).*

β-catenin

β -catenin expression was studied in 5 HB tumors that showed a strong expression of GS. Of these, 3 samples did not show appreciable cytoplasmic or nuclear accumulation of β-catenin. Figure 16 shows one of the β-catenin-positive tumors. The tumor contained CPS+/GS+, CPS+/GS-, and CPS-/GS+ nodules. β-catenin expression was confined to tumor cells and appeared to correlate with basophilia in the H&A staining. Within β-catenin-positive fields, isolated islands of cells showed strong nuclear accumulation of β-catenin (Figure 17). Nuclear accumulation did not appear to depend strongly on the cytosolic concentration of β-catenin (Figure 17). Furthermore, there was a poor correlation between GS and β-catenin expression (Figure 16).

Figure 16 (*see colour pages 147*) *Hepatoblastoma nodule in the liver of a 21 months-old female showing expansive growth. Panels A, B, and C, D are serial sections, with a few sections missing in between. Panel A is stained with H&A, panel B for the presence of β-catenin, panel C for carbamoyl phosphate synthetase and panel D for glutamine synthetase (GS). Note heterogeneity in staining between all panels, but especially between β-catenin and GS.*

Figure 17 (*see colour pages 148*) *Hepatoblastoma nodule in the liver of a 12 months-old male. The panels do not represent serial sections. Note difference in overall β-catenin accumulation and in degree of nuclear translocation. Also note extensive erythropoiesis.*

Discussion

Among the findings in the present study, the discrepancy between the usually more or less homogeneous phenotype of the cells within a nodule as opposed to pronounced heterogeneity between, often adjacent, nodules, in combination with expression patterns that, in aggregate, appeared to point to tumor progression, were most striking. Other interesting observations were the apparent correlation between the HB growth pattern and the response of the surrounding tissue and the poor correlation between β-catenin nuclear translocation and GS expression. Erythropoiesis was often seen and did not appear to correlate with specific gene expression patterns.

Growth pattern

More than half of the tumors exhibited an infiltrative growth pattern without a well-delineated border (see Figure 2). Conversely, a fibrous (pseudo)capsule (Figure 4) or a stretched appearance of the surrounding hepatocytes (Figure 3) that might suggest expansive growth was less often found. The accumulation of CK7 in liver lobules that had become trapped between tumor nodules (Figure 5) and strands of strongly CK19-positive epithelial cells in the thick, fibrotic sheaths surrounding nodules (not shown) suggest transdifferentiation into bile ductular structures. In addition, CK-positive cells may invade the connective tissue surrounding nodules (Figure 2). We therefore conclude that most HBs grow invasively, but that the fibroblastic response of the surrounding tissue may vary and affect growth rate.

Nodular development

The gene expression pattern of cells within nodules was remarkably homogeneous, whereas that between (adjacent) nodules might differ completely (e.g. Figure 13). In this respect, Figure 10 was instructive, showing a phenotypically different nodule within a larger, itself homogeneous nodule. These findings suggest a "founder" effect, that is, one or a few tumor cells apparently change their gene-expression program, possibly due to a mutation, and subsequently expand. Such a loss of expression of particular genes (relative to the parent nodule) reflects a dedifferentiation event. Genotypic instability is a property of most tumors, including HB[11,12,46]. This finding predicts that nodules of genotypically unstable HBs more quickly generate "daughter" nodules with a different phenotype than genotypically stable HBs. It may therefore be of interest to establish whether genotypic stability does indeed correlate with the size of phenotypically uniform nodules.

Gene-expression patterns

The reproducible exception to a homogeneous expression pattern in nodules was GS, which in many cases was only expressed in the nodular periphery (Figure 14). This localization of GS expression has also been described in humans with HCC[56]. In nodules in which GS is near-homogeneously expressed (Figures 6 and 7), a shallow, but reciprocal gradient in expression between CPS and GS can be discerned, with CPS being maximally expressed near the feeding vessels and GS away from them (Figure 7C,D). This finding is reminiscent of zonation in gene expression

and suggests that this zonation reflects gradients in e.g. oxygenation[27]. Indeed, GS expression has been shown to increase in hypoxic conditions[57]. The absence of CK7- or CK19-positive structures in this nodule is indicative of the "unpaired" arterial supply in of hepatic tumors ("Unpaired" arteries are not accompanied by a bile duct)[58].

Glutamine synthethase expression in the tumor shown in Figure 12 co-localizes with CK19, a typical portal marker. In the embryo, the development of bile ducts from hepatoblasts depends on the interaction of the hepatoblasts with the connective-tissue cells of the portal tract[59]. The near-absence of portal tracts in the nodule shown in Figure 7 may explain the absence of CK7- and CK19-positive cells. In all likelihood, therefore, expression of CK7 and CK19 as markers for bile-ductular structures is confined to epithelial cells that are in close contact with a sufficient number of connective-tissue cells. Such a mechanism is compatible with the confinement of CK7 and CK19 expression to the outer periphery of the nodules shown in Figures 6 and 14. Although the co-localization of GS and CK7 or CK19 is in apparent contradiction with the concept of zonation of gene expression in the liver, it appears to reflect the simultaneous effects of 2 independent mechanisms that are responsible for the phenotypic differences of the epithelial cells in the liver lobule, viz. epithelio-mesenchymal interactions and porto-central gradients in metabolites and signaling factors. In conjunction with the appearance of "unpaired" arteries and the disappearance of the wide portal tracts, these findings imply that nodules at this stage of development are still fully responsive to environmental stimuli. As such, zonation of gene expression in nodules may therefore be useful as a marker for tumor progression.

GS and β-catenin

We studied the relation between GS and β-catenin expression, because GS is a downstream target of cellular β-catenin accumulation[48] and because stabilizing amino-terminal mutations of the β-catenin gene are often seen in advanced HB[46,47]. Somewhat unexpectedly, β-catenin expression was markedly heterogeneous itself (Figures 16 and 17), even in areas that were strongly GS-positive, while some strongly β-catenin-positive nodules did not express GS. These observations suggest that there is no obligatory relationship between nuclear translocation of β-catenin and GS (over-)expression. In agreement with this conclusion, we found 3 tumors in which β-catenin was hardly activated, even though GS was strongly expressed. We hypothesize that in these cases pericentral environmental conditions that facilitate GS expression prevail. In any case, we have to conclude that GS expression cannot serve as a surrogate marker for the activation and nuclear translocation of β-catenin.

Cytokeratins as markers for stem cells

Even though many cells in HB tumors express stem cell markers, these markers usually don't identify a specific or identifiable population of cells[21,34,60]. We studied CK7 and CK19 expression. In normal development, the expression of CK19 becomes confined to the biliary tree at approximately 10 weeks of gestation[61], whereas CK7 expression becomes detectable only at approx. 20 weeks of gestation[62]. In our hands, CK19 expression was confined to a smaller population of ductular

cells than CK7 (Figure 1), suggesting CK7 stained the canals of Hering (cf. ref.[63]). Hence, the comparison of CK19 and CK7 might identify a persisting stem cell-like population. The expression of CK19 and CK7 in HB has indeed been described[21,34,37-39], but whether or not such cells represent HB stem cells is still debated. We observed CK staining in epithelial cells that were "trapped" in the fibroblastic fascia-like structure that surrounded tumor nodules in many patients. Although these cells may express stem-cell markers[21], they appeared solidly epithelial. We also observed expression of CK7 and -19 in the nodular periphery, but consider this, as explained in a previous paragraph, a consequence of the topographical position of the cells (cf. ref.[64]). Finally, we did observe no CK7 in any of the tumors investigated and CK19 in only 3 out of 14 tumors investigated. In one of these cases, tumor cells looked undifferentiated (Figure 3), but it is questionable whether these cells qualify as stem cells. If CK19-positive cells indeed represent tumor stem cells, they would not be a regular feature of hepatoblastoma and would be confined to the tumoral periphery.

Conclusions

Several studies have proposed new prognostic factors that are based on the phenotypic properties of the tumor cells within a hepatoblastoma. Other studies have identified stem cell markers, tumor markers, and prognostic factors in HB. In the present study, we identified some characteristics in the growth pattern (expansive vs. infiltrative), the size of phenotypically homogeneous nodules (which appears to reflect the rate of dedifferentiation), the vascularization pattern (the development of perfusion by an "unpaired" central artery) and the zonation of gene expression in the nodules that were not yet been studied as potential prognostic markers, but appear to be promising in this respect.

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References

1. Vos A. Primary liver tumours in children. *Eur J Surg Oncol* 1995; 21(1):101-5.
2. Perilongo G, Shafford EA. Liver tumours. *Eur J Cancer* 1999; 35(6):953-8; discussion 958-9.
3. Schnater JM, Köhler SE, Lamers WH, Von Schweinitz D, Aronson DC. Where do we stand with hepatoblastoma? a review. *Cancer* 2003; 98(4):668-78.
4. Czauderna P, Mackinlay G, Perilongo G, Brown J, Shafford E, Aronson D, et al. Hepatocellular carcinoma in children: results of the first prospective study of the international society of pediatric oncology group. *J Clin Oncol* 2002; 20:2798-2804.
5. Katzenstein HM, Krailo MD, Malogolowkin MH, Ortega JA, Lin-Mu Y, Douglass EC, et al. Hepatocellular carcinoma in children and adolescents: results from the Pediatric Oncology Group and the Children's Cancer Group intergroup study. *J Clin Oncol* 2003; 20(12):2789-97.
6. Schnater JM, Kuijper CF, Zsiros J, Heij HA, Aronson DC. Preoperative diagnostic biopsy and surgery in paediatric liver tumours - The Amsterdam experience -. *Eur J Surg Oncol* 2005; 31(10):1160-5.
7. von Schweinitz D, Hecker H, Schmidt-von-Arndt G, Harms D. Prognostic factors and staging systems in childhood hepatoblastoma. *Int J Cancer* 1997; 74(6):593-9.
8. Brown J, Perilongo G, Shafford E, Keeling J, Pritchard J, Brock P, et al. Pretreatment prognostic factors for children with hepatoblastoma-- results from the International Society of Paediatric Oncology (SIOP) study SIOPEL 1. *Eur J Cancer* 2000; 36(11):1418-25.
9. Perilongo G, Brown J, Shafford E, Brock P, De Camargo B, Keeling JW, et al. Hepatoblastoma presenting with lung metastases. *Cancer* 2000; 89(8):1845-53.
10. Steenman M, Tomlinson G, Westerveld A, Mannens M. Comparative genomic hybridization analysis of hepatoblastomas: additional evidence for a genetic link with Wilms tumor and rhabdomyosarcoma. *Cytogenet Cell Genet* 1999; 86(2):157-61.
11. Hu J, Wills M, Baker BA, Perlman EJ. Comparative genomic hybridization analysis of hepatoblastomas. *Genes Chromosomes Cancer* 2000; 27(2):196-201.
12. Yamada S-I, Ohira M, Horie H, Ando K, Takayasu H, Suzuki Y, et al. Exression profiling and differential screening between hepatoblastomas and the corresponding normal livers: identification of high expression of the PLK1 oncogene as a poor-prognostic indicator of hepatoblastomas. *Oncogene* 2004; 23:5901-11.
13. Dehner LP, Manivel JC. Hepatoblastoma: an analysis of the relationship between morphologic subtypes and prognosis. *Am J Pediatr Hematol Oncol* 1988; 10(4):301-7.
14. Haas JE, Muczynski KA, Krailo M, Ablin A, Land V, Vietti TJ, et al. Histopathology and prognosis in childhood hepatoblastoma and hepatocarcinoma. *Cancer* 1989; 64(5):1082-95.
15. Conran RM, Hitchcock CL, Waclawiw MA, Stocker JT, Ishak KG. Hepatoblastoma: the prognostic significance of histologic type. *Pediatr Pathol* 1992; 12(2):167-83.
16. von Schweinitz D, Wischmeyer P, Leuschner I, Schmidt D, Wittekind C, Harms D, et al. Clinico-pathological criteria with prognostic relevance in hepatoblastoma. *Eur J Cancer* 1994; 8(8):1052-8.
17. Fasano M, Theise ND, Nalesnik M, Goswami S, Garcia de Davila MT, Finegold MJ, et al. Immunohistochemical evaluation of hepatoblastomas with use of the hepatocyte-specific marker, hepatocyte paraffin 1, and the polyclonal anti-carcinoembryonic antigen. *Mod Pathol* 1998; 11(10):934-8.
18. Haas JE, Feusner JH, Finegold MJ. Small cell undifferentiated histology in hepatoblastoma may be unfavorable. *Cancer* 2001; 92(12):3130-4.
19. Ruck P, Xiao JC, Pietsch T, Von Schweinitz D, Kaiserling E. Hepatic stem-like cells in hepatoblastoma: expression of cytokeratin 7, albumin and oval cell associated antigens detected by OV-1 and OV-6. *Histopathology* 1997; 31(4):324-9.
20. Ruck P, Xiao JC. Stem-like cells in hepatoblastoma. *Med Pediatr Oncol* 2002; 39(5):504-7.
21. Fiegel HC, Gluër S, Roth B, Rischewski J, von Schweinitz D, Ure B, et al. Stem-like cells in human hepatoblastoma. *J Histochem Cytochem* 2004; 52:1495-1501.
22. Lincoln DT, Temmin L, al-Jarallah MA, Sinowatz F, Gabius H, Hifnawi el S, et al. Potential usefulness of biotinylated neoglycoproteins as tumor markers. *Nutrition* 1995; 11(5 Suppl):632-7.

23. Van Tornout JM, Buckley JD, Quinn JJ, Feusner JH, Krailo MD, King DR, et al. Timing and magnitude of decline in alpha-fetoprotein levels in treated children with unresectable or metastatic hepatoblastoma are predictors of outcome: a report from the Children's Cancer Group. *J Clin Oncol* 1997; 15(3):1190-7.
24. Schnater JM, Hakvoort TBM, de Krom M, Vermeulen JLM, Bras J, Von Schweinitz D, et al. Are these the new molecular markers in liver tumours? *Eur J Gastroenterol Hepatol* 1998; 10(12):A88.
25. Gregory JJ, Jr., Finlay JL. Alpha-fetoprotein and beta-human chorionic gonadotropin: their clinical significance as tumour markers. *Drugs* 1999; 57(4):463-7.
26. von Schweinitz D, Kraus JA, Albrecht S, Koch A, Fuchs J, Pietsch T. Prognostic impact of molecular genetic alterations in hepatoblastoma. *Med Pediatr Oncol* 2002; 38:104-8.
27. Meijer AJ, Lamers WH, Chamuleau RAFM. Nitrogen metabolism and ornithine cycle function. *Physiol Rev* 1990; 70:701-48.
28. Dingemanse MA, de Boer PAJ, Mori M, Lamers WH, Moorman AFM. Development of the ornithine cycle in rat liver: zonation of a metabolic pathway. *Hepatology* 1996; 24:407-11.
29. Dingemanse MA, Lamers WH. Expression patterns of ammonia-metabolizing enzymes in the liver, mesonephros, and gut of human embryos and their possible implications. *Anat Rec* 1994; 238(4):480-90.
30. Lamers WH, Zonneveld D, Charles R. Inducibility of carbamoylphosphate synthetase (ammonia) in cultures of embryonic hepatocytes: Ontogenesis of the responsiveness to hormones. *Develop Biol* 1984; 105(2):500-8.
31. Koike T, Shiojiri N. Differentiation of the mouse hepatic primordium cultured in vitro. *Differentiation* 1996; 61(1):35-43.
32. Yin L, Lynch D, Sell S. Participation of different cell types in the restitutive response of the rat liver to periportal injury induced by allyl alcohol. *J Hepatolog* 1999; 31(3):497-507.
33. Yin L, Lynch D, Ilic Z, Sell S. Proliferation and differentiation of ductular progenitor cells and littoral cells during the regeneration of the rat liver to CCl4/2-AAF injury. *Histol Histopathol* 2002; 17(1):65-81.
34. Ruck P, Xiao JC, Kaiserling E. Small epithelial cells and the histogenesis of hepatoblastoma. Electron microscopic, immunoelectron microscopic, and immunohistochemical findings. *Am J Pathol* 1996; 148(1):321-9.
35. Vessey CJ, De La Hall PM. Hepatic stem cells: a review. *Pathology* 2001; 33:130-41.
36. Qin AL, Zhou XQ, Zhang W, Yu H, Xie Q. Characterization and enrichment of hepatic progenitor cells in adult rat liver. *World J Gastroenterol* 2004; 10(10):1480-6.
37. van Eyken P, Sciot R, Paterson A, Callea F, Kew MC, Desmet VJ. Cytokeratin expression in hepatocellular carcinoma: an immunohistochemical study. *Hum Pathol* 1988; 19(5):562-8.
38. Van Eyken P, Sciot R, Callea F, Ramaekers F, Schaart G, Desmet VJ. A cytokeratin-immunohistochemical study of hepatoblastoma. *Hum Pathol* 1990; 21(3):302-8.
39. Pontisso P, Barzon M, Basso G, Cecchetto G, Perilongo G, Alberti A. Cytokeratins patterns in childhood primary liver tumors. *Int J Clin Lab Res* 1993; 23(4):225-7.
40. Roskams T. Different types of liver progenitor cells and their niches. *J Hepatol* 2006; 45(1): 1-4.
41. Koch A, Denkhaus D, Albrecht S, Leuschner I, von Schweinitz D, Pietsch T. Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the beta-catenin gene. *Cancer Res* 1999; 59(2): 269-73.
42. Wei Y, Fabre M, Branchereau S, Gauthier F, Perilongo G, Buendia M-A. Activation of beta-catenin in epithelial and mesenchymal heptoblastomas. *Oncogene* 2000; 19:498-505.
43. Takayasu H, Horie H, Hiyama E, Matsunaga Y, Hayashi Y, Watanabe Y, et al. Frequent deletions and mutations of the beta-catenin gene are associated with overexpression of cyclin D1 and fibronectin and poorly differentiated histology in childhood hepatoblastoma. *Clin Cancer Res* 2001; 7:901-8.
44. Udatsu Y, Kusafuka T, Kuroda S, Miao J, Okada A. High frequency of betacatenin mutations in hepatoblastoma. *Pediatr Surg Int* 2001; 17:508-12.
45. Taniguchi K, Roberts LR, Aderca IN, Dong X, Qian C, Murphy LM, et al. Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. *Oncogene* 2002; 21:4863-71.
46. Buendia MA. Genetic alterations in hepatoblastoma and hepatocellular carcinoma: common and distinctive aspects. *Med Pediatr Oncol* 2002; 39(5):530-5.
47. Lee HC, Kim M, Wands JR. Wnt/Frizzled signaling in hepatocellular carcinoma. *Front Biosc* 2006; 11: 1901-5.
48. Cadoret A, Ovejero C, Terris B, Souil E, Lévy L, Lamers WH, et al. New targets of beta-catenin signaling in the liver are involved in glutamine metabolism. *Oncogene* 2002; 21:8293-8301.

49. Moorman AF, Vermeulen JL, Charles R, Lamers WH. Localization of ammonia-metabolizing enzymes in human liver: ontogenesis and heterogeneity. *Hepatology* 1989; 9(3):367-72.
50. Christa L, Simon M-T, Flinois J-P, Gebhardt R, Brechot C, Lassere C. Overexpression of glutamine synthetase in human primary liver cancer. *Gastroenterology* 1994; 106:1312-20.
51. Osada T, Sakamoto M, Nagawa H, Yamamoto J, Matsumo Y, Iwamatsu A, et al. Acquisition of glutamine synthetase expression in human hepatocarcinogenesis: relation to disease recurrence and possible regulation by ubiquitin-dependent proteolysis. *Cancer* 1999; 85(4):819-31.
52. Osada T, Nagashima I, Tsuno NH, Kitayama J, Nagawa H. Prognostic significance of glutamine synthetase expression in unifocal advanced hepatocellular carcinoma. *J Hepatol* 2000; 33(2):247-53.
53. Desdouets C, Fabre M, Gauthier F, Brechot C, Sobczak-Thepot J. Proliferation and differentiation of a human hepatoblastoma transplanted in the Nude mouse. *J Hepatol* 1995; 23(5):569-77.
54. Charles R, de Graaf A, Moorman AF. Radioimmunochemical determination of carbamoyl-phosphate synthetase (ammonia) content of adult rat liver. *Biochim Biophys Acta* 1980; 692(1):36-49.
55. Lamers WH, Vermeulen JLM, Hakvoort TBM, Moorman AFM. Expression pattern of glutamine synthetase marks transition from collecting into conducting hepatic veins. *J Histochem Cytochem* 1999; 47(12):1507-12.
56. Hsia CC, Evarts RP, Nakatsukasa H, Marsden ER, Thorgeirsson SS. Occurrence of oval-type cells in hepatitis B virus-associated human hepatocarcinogenesis. *Hepatology* 1992; 16:1327-33.
57. Kobayashi S, Millhorn DE. Hypoxia regulates glutamate metabolism and membrane transport in rat PC12 cells. *J Neurochem* 2001; 76(6):1935-48.
58. Park YN, Kim YB, Yang KM, Park C. Increased expression of vascular endothelial growth factor and angiogenesis in the early stage of multistep hepatocarcinogenesis. *Arch Pathol Lab Med* 2000; 124(7):1061-65.
59. Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. *Gastroenterology* 2004; 127(6):1775-86.
60. Badve S, Logdberg l, Lal A, de Davila MT, Greco MA, Mitsudo S, et al. Small cells in hepatoblastoma lack "oval" cell phenotype. *Mod Pathol* 2003; 16(9):930-6.
61. Stosiek P, Kapser M, Karsten U. Expression of cytokeratin 19 during human liver organogenesis. *Liver* 1990; 10:59.
62. van Eyken P, Sciot R, Callea F, Van der Steen K, Moerman P, Desmet VJ. The development of the intrahepatic bile ducts in man: a keratin-immunohistochemical study. *Hepatology* 1988; 8(6):1586-95.
63. Saxena R, Theise N. Canals of Hering: recent insights and current knowledge. *Semin Liver Dis* 2004; 24(1): 43-8.
64. Zimmermann A. The emerging family of hepatoblastoma tumours: From ontogenesis to oncogenesis. *Eur J Cancer* 2005; 41(11):1503-14.

'Understanding the biological aspects of hepatoblastoma is useful if one wants to improve the outcome for the 25% of the patients who still die due to their disease'

'But for testing additional treatment options, a relevant and reproducible animal model is lacking!'

Chapter 6

Subcutaneous and intrahepatic growth of human hepatoblastoma

In immunodeficient mice

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Abstract

Background: Hepatoblastoma is the most frequent malignant pediatric liver tumor. Approximately 25% of hepatoblastoma patients cannot be cured with current treatment protocols. Additional treatment options must, therefore, be developed. Subcutaneous animal models for hepatoblastoma exist, but a more physiologic intrahepatic model is lacking.

Methods: The α-fetoprotein-expressing hepatoblastoma-cell lines HepT1, HuH6 and the childhood hepatocellular carcinoma-cell line HepG2 were injected subcutaneously and intrasplenically into HsdCpb:NMRI-Foxn1^{nu} mice. Tumor growth was monitored by measuring tumor size for subcutaneous and serum human α-fetoprotein levels for intra-abdominal tumors. Tumors were characterized microscopically.

Results: Subcutaneous tumor growth occurred in 70% (7/10) of mice injected with HuH6 and 50% (5/10) of mice injected with HepG2. HepT1 did not form tumors. Accumulation of serum α-fetoprotein reflected tumor growth. Intrasplenic growth was seen in 52% (14/27, HuH6) and 10% (3/30, HepG2) of the mice, with only HuH6 forming intrahepatic tumors in 26% (7/27) of the mice. Growth pattern and α-fetoprotein production were similar at the subcutaneous and intra-abdominal location. Intrahepatic grafting occurred by metastatic spread from the spleen, produced well-defined nodules, and was accompanied by a weakened expression of the hepatocyte marker carbamoylphosphate synthetase, and the canalicular markers CD10 and cytokeratin7. The expression of cytokeratin18 and –19, active caspase3, and β-catenin was increased. There were no lung metastases.

Conclusion: We established an intrahepatic mouse model for human hepatoblastoma, in which tumor growth could be monitored by serum α-fetoprotein levels. Engrafting in the liver occurred by metastatic spread from the spleen and was accompanied by some loss of differentiation features.

Introduction

Hepatoblastoma (HB) and hepatocellular carcinoma (HCC) are the most common malignant liver tumors in childhood[1]. A high serum α-fetoprotein level is found in 90-95% of HB and in 60-90% of HCC patients[2]. α-Fetoprotein is, therefore, used clinically to monitor treatment[3]. Chemotherapy to reduce tumor size preoperatively, in combination with radical surgery, has dramatically improved the prognosis for HB, but that for HCC remains poor[2]. Currently, complete removal of the tumor is achieved in 75% (HB) and 40% (HCC) of affected children[4-7]. This outcome still leaves a large proportion of children with an unfavorable prognosis and makes the development of novel therapeutic strategies highly desirable.

A relevant and reproducible animal model is a necessary tool to test novel treatments. Such an animal model ideally harbors tumors that resemble the original tumor with respect to localization and biology. Many human HCC cell lines grow subcutaneously, but few intrahepatic xenograft models exist[8,9]. These findings suggest that the requirements for intrahepatic tumor growth are more stringent than those for subcutaneous tumor growth. It has proven difficult to grow HB subcutaneously and[10-15], to our knowledge, no intrahepatic model is available. We have, therefore, transplanted the well-characterized HB cell lines HuH6 and HepT1 that were previously shown to grow subcutaneously to the spleen[16,17]. From here, tumor cells can populate the liver directly by "overflow" into the splenic vein or later by metastatic spread. The childhood HCC cell line HepG2 was included as a HCC reference. All cell lines secrete α-fetoprotein, so that tumor growth could be established non-invasively. We report a remarkably different capacity of the respective cell lines to give rise to tumors in vivo. HepT1 cells did not grow in vivo, even in a subcutaneous location, whereas more HuH6 and HepG2 cells were required to establish nodules in the spleen than in the subcutis. Only HuH6 was able to settle in the liver. We have examined the intrahepatic HuH6 tumor nodules and observed that they develop by metastatic spread from the spleen.

Material and methods

Cell cultures

The human HB cell lines HepT1 and HuH6 (Japanese Collection of Research Biosources, Osaka, Japan)[16-18], and the human HCC cell line HepG2 (ATCC, Rockville, MD) were cultured in RPMI medium (Biochrom)[19], 10% fetal bovine serum, and 2mM L-glutamine (Invitrogen). Cells were regularly checked for the presence of mycoplasma using the VenorGeM kit (Minerva Biolabs, Germany).

Animals

HsdCpb:NMRI-Foxn1^{nu} mice, which carry the nude gene in an outbred Swiss NMRI background, were purchased from Harlan, The Netherlands[20,21]. These mice are congenitally athymic, therefore lack T-cells and show reduced xenograft rejection[22]. Cells were injected in 6-to-7 week old female mice (24-30g), kept in filter-top cages at 22°C, 60% humidity. Sterilized food and water were accessible ad libitum. All animal experiments were approved by the Swiss ethical committee (Kantonales Veterinäramt, Basel).

Subcutaneous injection of tumor cells

Subconfluent cultures of HepT1, HuH6 and HepG2 were trypsinized, suspended at 5x10^6 cells/mL and stored on ice for injection. Ten mice were injected subcutaneously in the left flank with 10^6 cells. Ten additional mice received 10^7 HepT1 cells. Tumor size was estimated weekly[14]. Mice were killed with CO_2/O_2 when tumors reached a diameter of 2 cm. A portion of each tumor was fixed in 4% buffered formaldehyde and processed for histological analysis, while another portion was frozen in liquid nitrogen and stored at −70°C.

Intra-splenic injection of tumor cells

Subconfluent monolayer cultures were trypsinized and stored on ice till injection. Mice were anesthetized with 7mL/Kg body weight Hypnorm (0.315mg/mL fentanylcitrate, 10mg/mL Fluanison), Dormicum (5mg/mL midazolam HCl) and H_2O (1:1:2) and operated on a 43°C (pre)warmed operation table. A suspension of 1x10^6 or 3x10^6 cells in 200μL RPMI medium was injected via a small median laparotomy into the lower pole of the spleen using a 30G needle (See also Appendix II). Six control mice were injected with 200μL 0.9% NaCl. Postoperatively, mice were kept warm and returned to their cages when fully awake.

In total, 11, 10, and 12 mice were injected intrasplenically with 1x10^6 HepG2, HuH6, and HepT1 cells, respectively, while 30, 27, and 10 mice were injected with 3x10^6 HepG2, HuH6, and HepT1 cells, respectively. At 7, 9, 11 and 15 weeks after injection, 2-3 mice per cell line injected with 1x10^6 cells were sacrificed, and liver, spleen and lungs were collected. Mice injected with 3x10^6 cells were sacrificed when >20U/ml human α-fetoprotein (hα-FP) was detected in tail-vein plasma. The volume of the tumor nodule(s) was estimated as described[23]. Specimens were fixed in 4% formaldehyde, stained with hematoxylin and eosin, and analyzed by the pathologist (*EB*). Distant metastases, particularly lung metastases, were looked for macroscopically and microscopically.

Alpha-fetoprotein expression

The expression of hα-FP in cells was determined by Western-blot analysis of cell lysates with rabbit anti-human α-fetoprotein antiserum (Dako, 1:800) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Transduction Laboratories), or by radioimmunoassay of cytosol preparations and culture medium (RIA-gnost AFP, Cis-Bio International, Schering, Switzerland). Both assays are specific for hα-FP. Culture medium was collected after 4 days and stored at −70°C.

Circulating α-fetoprotein levels were determined by radioimmunoassay in plasma. Human lung fibroblasts were used as negative and human umbilical serum (collected after informed consent) as positive control.

Immunohistochemistry

Hepatoblastoma foci in spleen and liver were stained for the expression of carbamoyl-phosphate synthetase (CPS; rabbit antiserum)[24], glutamine synthetase (GS) and β-catenin (mouse monoclonals, Transduction Laboratories, Lexington, KY), OV6 (oval-cell marker; polyclonal antiserum)[25], cytokeratin7, cytokeratin18 (mouse monoclonal, Progen Biotechnik GmbH, Heidelberg, Germany), cytokeratin19 (mouse IgG1 antibody; Novus Biologicals; Littleton, CO), caspase3 (affinity-purified anti-human/mouse "active" caspase3; R&D systems, Minneapolis, MN)[26], BCL2 (mouse IgG1 anti-human BCL2; DAKO, Glostrup, Denmark), CD10 and the hepatocyte-growth factor receptor c-MET (mouse-monoclonal 3D4; Zymed, Invitrogen, Breda, Netherlands). Antibody binding was visualized with goat-anti-mouse or -anti-rabbit serum coupled to horseradish peroxidase (Transduction Laboratories). Where necessary (c-Met, β-catenin) the MOMTM kit (Vector Laboratories, Peterborough, U.K.) was used to suppress non-specific binding of secondary anti-mouse antibodies. Cell proliferation was assessed by intraperitoneal injection of 1.5mg bromodeoxyuridine (150μL 10mg/mL) 16 and 4 hours before sacrifice and visualized with monoclonal antibody and detection kit (Roche Diagnostics GmbH, Mannheim, Germany).

Results

The HB cell lines HepT1 and HuH6, and the childhood HCC cell line HepG2 showed comparable growth rates *in vitro,* with cell doubling times for HuH6 and HepG2 of 1.1 and 1.4 days, respectively. We confirmed that all three cell lines produced α-fetoprotein, with HepG2 being approx. 20-fold more active than HuH6 and HepT1 (not shown; cf. ref. 27)[27]. The morphologic characteristics of the respective cell lines *in vitro* have been described[13,16,17]. We further established that HuH6 cells expressed the oval-cell marker OV6[25], the mature hepatocyte markers CPS and GS[28,29], the hepatic stem-cell and cholangiocyte marker cytokeratin19[30], and the apoptosis marker "activated" caspase3 (Figure 1)[26]. The anti-apoptosis marker BCL2 was not expressed[31], while the hepatocyte-growth factor receptor c-MET was expressed at low levels[17]. Expression of β-catenin was remarkably heterogeneous.

Figure 1 (*see colour pages 149*) E*xpression of the oval-cell marker OV6 (A), glutamine synthetase (B), carbamoylphosphate synthetase (C), cytokeratin19 (D), hepatocyte-growth factor-receptor c-MET (E), anti-apoptosis-marker BCL2 (F), and apoptosis-marker "active" caspase3 (G), and β-catenin (H) in HuH6 cells in culture. Panel I: negative controls, incubated with goat-anti-mouse IgG.*

Tumor growth upon subcutaneous injection

HepT1 cells did not grow subcutaneously, whereas HuH6 and HepG2 had a take rate of 70% and 50%, respectively. Local tumor growth was detectable from 3.5 and 4.5 weeks after injection for HuH6 and HepG2 cells, respectively. HuH6 tumors grew as light-gray, solid nodules within a well-developed fibrous capsule, whereas HepG2 tumors showed the typical dark-blue aspect and were soft. Distant metastases were not found. Subcutaneously, HuH6 and HepG2 grew at a similar rate but compared to *in vitro*, their doubling time had increased to 5.4±0.5 and 9.7±0.8 days, respectively (N = 3 for both cell lines). Hα-FP became detectable in tail-vein blood when a visible tumor appeared subcutaneously, whereas non-tumor bearing mice and mice injected with HepT1 cells were hα-FP-negative. Plasma hα-FP levels in HepG2-bearing mice (>1,000 IU/mL) were higher than those in HuH6-bearing mice (range 120-560 IU/mL), reflecting the difference in hα-AP production *in vitro* (Table 1).

Table 1 *Experimental setup of subcutaneous injection. Number of mice injected with each cell type, number of cells injected, α-FP concentrations, and number of mice that developed subcutaneous (s.c.) tumors and the take rate in % are listed*

Cell type (Tumor)	no. mice	no. cells	s.c. tumors (%)	AFP (U/ml)
HepT1 (HB)	10	10^6	0	0
HepT1 (HB)	10	10^7	0	0
HepG2 (HCC)	10	10^6	5 (50)	>1000
HuH6 (HB)	10	10^6	7 (70)	120-560

HB: hepatoblastoma; HCC: hepatocellular carcinoma.

Microscopically, HuH6 tumor morphology was consistent with an embryonal HB, showing trabeculae of small hyperchromatic cells with little cytoplasm and focal pseudoglandular aggregates interspersed with areas of necrosis (Figure 2A). Erythropoietic foci were not evident. HepG2 nodules were composed of trabecular tumor-cell aggregates that were rich in cytoplasm and showed nuclear pleomorphism and prominent nucleoli (Figure 2B). Areas of hemorrhagic necrosis were prominent.

Figure 2 (*see colour pages 150*) *Histological characteristics of a HuH6 (A) and a HepG2 (B) subcutaneous tumor. Bromodeoxyuridine incorporation into HuH6 tumors in the spleen (C) and liver (D). Inflammatory cell nest (E) and hepatocellular mitosis (F; circles) in a liver with a HuH6 nodule. H&E stain. Bar: 50 µm.*

Tumor growth upon intrasplenic injection

HepT1 injected mice did not develop tumors. Two out of 11 mice injected with $1x10^6$ HepG2 cells developed a tumor in the spleen, but none in the liver. Hα-FP was detected in tail-vein plasma of these 2 mice from week 7 onwards (230 and 7,000 IU/mL). When $1x10^6$ HuH6 cells were injected, only one mouse (10%) developed a tumor in the liver, with 210 IU hα-FP/mL plasma at 9 weeks. Hα-FP was not detectable in the final, large blood sample of mice injected with HepT1 or 0.9% NaCl (Table 2).

Table 2 *Experimental setup of intrasplenic injection. Number of mice injected with each cell type, number of cells injected, α-FP concentrations, and number of mice that developed intrasplenic (i.s.) or intrahepatic (i.h.) tumors and the take rate in % are listed*

Cell type (Tumor)	no. mice	no. cells	tumors (%)	AFP (U/ml)	
			i.s	i.h.	
HepT1 (HB)	12	10^6	0	0	0
HepT1 (HB)	10	$3x10^6$	0	0	0
HepG2 (HCC)	11	10^6	2 (18)	0	230, 7000
HepG2 (HCC)	30	$3x10^6$	3 (10)	0	50-1000
HuH6 (HB)	10	10^6	0	1 (10)	210
HuH6 (HB)	27	$3x10^6$	14 (52)	7 (26)	5-900

HB: hepatoblastoma; HCC: hepatocellular carcinoma.

Injection of $3x10^6$ cells yielded elevated plasma hα-FP concentrations after 4-6 weeks in 14 mice (52%) injected with HuH6 (range: 5-900 IU/mL) and in 3 mice (10%) injected with HepG2 (range: 50-1,000 IU/mL). Serial measurements of hα-FP were available for 5 HuH6-injected mice and 2 HepG2-injected mice. The doubling time of plasma hα-FP concentration in these mice was 5.7±0.7 days for HuH6 tumors and 6.9±0.5 days for HepG2. No new cases with elevated hα-FP were found later than 6 weeks after transplantation. At autopsy, tumors were only found in mice with an elevated plasma hα-FP level. In the 14 HuH6-injected mice with elevated hα-FP levels, a solitary tumor nodule was found in the spleen of all 14 and mostly multifocal tumors in the liver of 7 mice. The mean volume of the intrasplenic tumors was 760 mm^3 (range: 90-1,960 mm^3), liver nodules ranged from microscopically visible to 1,000 mm^3. In the 3 HepG2-injected mice with an elevated hα-FP level, tumors were only found in the spleen (range: 160-1,400 mm^3). Again, HepT1-injected mice did not develop tumors (Table 2). Distant metastases were not found.

Phenotypic characterization of intra-abdominal HuH6 tumors

The microscopic anatomy of the subcutaneous and abdominal HuH6 tumors was consistent with HB of the embryonal epithelial subtype, without a mesenchymal component. Subcutaneous HuH6 tumors were characterized by a predominantly trabecular growth pattern. Both intrasplenically and intrahepatically, HuH6 tumors showed a slightly different architecture with a predominantly solid-cystic growth pattern, slightly smaller individual tumor cells, and irregular pseudoglandular structures, as also seen in human embryonal hepatoblastoma[32]. Tumors were surrounded by an inconspicuous, thin pseudocapsule. Bromodeoxyuridine incorporation showed that DNA synthesis in tumor cells was more active in spleen than in liver, whereas the surrounding tissue, especially hepatocytes, remained unlabeled (Figure 2C, D). The liver of tumor-bearing animals showed occasional foci of inflammatory cells (Figure 2E) and hepatocyte mitoses (Figure 2F) without evidence of nearby nodules. The hepatic tumor nodules apparently developed as metastases, as tumors were seen invading vessels in the spleen (Figure 3$C_{1, 2}$) and tumor thrombi were found in small portal-vein branches (Figure 4C).

We tested the tumors for the expression of α-fetoprotein, OV-6 and GS, all typically overexpressed in hepatoblastoma. The hα-FP antiserum stained the HuH6 tumor in both spleen and liver (Figure 3A, B). The oval-cell marker OV-6 was expressed in HuH6 cells in the spleen, with some variability of staining intensity (Figure 3C). In the liver, all nodules stained positive for OV-6 (Figure 3D). GS expression in both intrasplenic and intrahepatic nodules was weak compared to that in pericentral hepatocytes (Figure 3E, F).

Figure 3 (*see colour pages 150*) *α-Fetoprotein (A,B), OV6 (C,D) and glutamine synthetase ((GS); E,F) expression in HuH6 tumor in the spleen (A,C,E) and liver (B,D,F). Panel A shows weakly positive and negative areas inside the tumor and panel B a weakly positive, intrahepatic microcystic tumor with a smooth, thin pseudocapsule. Panels C1 and C2 show weakly OV6-positive tumor aggregate (asterisk) invading splenic vessel (brown, due to erythrocyte-catalase activity), whereas panel D shows OV6-positive cystic tumor in liver. Panels E and F show weakly GS-positive tumor tissue in spleen and liver and, for comparison, strongly positive hepatocytes (brown) surrounding a central vein (thick arrow). Tumor tissue is marked by an asterisk, host spleen and liver tissue by a thin arrow. Bar A-C: 200 μm, bar D-F 100 μm.*

The mature-hepatocyte marker CPS was expressed in some but not all nodules in the spleen (Figure 4A), but its expression was very weak in all intrahepatic nodules (Figure 4B-D). Cytokeratin7, which is expressed in hepatic stem cells, HB, and cholangiocarcinoma, but infrequently in HCC[30,33], was only weakly expressed in the intrasplenic nodules and absent from nodules in the liver (Figure 5A, B). Cytokeratin18 and -19 were strongly expressed in intrasplenic, and even stronger in intrahepatic tumors (Figure 5C-F).

Figure 4 (*see colour pages 151*) *Carbamoylphosphate synthetase (CPS) expression in HuH6 tumor in the spleen (A) and liver (B-D). The intrasplenic tumor expresses the enzyme, whereas the small (B) and large (C) intraportal metastases hardly express the protein. All host-hepatocytes except those directly surrounding a central vein (C; arrows) express CPS. Note flattening of (CPS-positive) host-hepatocytes surrounding the tumor (arrows), indicating growth by expansion (D). Tumor tissue is marked by an asterisk, host tissue by a thin arrow. Bar A, B: 200 µm, bar C: 100 µm, bar D: 50 µm.*

Figure 5 (*see colour pages 152*) *Cytokeratin7 (A,B), cytokeratin18 (C,D), and cytokeratin19 (E,F) expression in HuH6 tumor in the spleen (A,C,E) and liver (B,D,F). Cytokeratin7 is weakly expressed in the intrasplenic and not in intrahepatic tumors, whereas cytokeratin18 and especially cytokeratin19 are strongly expressed in tumor cells in both spleen and liver. Bar: 200 µm.*

As demonstrated by the bile canalicular marker CD10, HuH6 cells were no longer polarized (Figure 6A, B). The hepatocyte-growth factor-receptor c-MET was strongly expressed at relatively high levels in HuH6 cells in the spleen and liver (Figure 6C, D). The spleen and liver tissue itself did not stain appreciably. HuH6 cells show remarkably heterogenous β-catenin staining (Figure 1H), mostly in the cell membranes. The intrasplenic ant intrahepatic tumors also show membrane staining and, in addition, a heterogenous, but overall very strong nuclear staining. The mouse spleen and liver only showed membranous staining (Figure 6E, F). Its expression was markedly higher in the hepatocytes surrounding the tumor than in the intrahepatic HuH6 cells themselves

Figure 6 (*see colour pages 152*) *CD10 (A,B), c-MET (C,D), and β-catenin (E,F) expression in HuH6 tumor in the spleen (A,C) and liver (B,D). Note (near) absence of CD10 and tumor capsule in liver tumor (B,D). Asterisk marks tumor tissue, host spleen and liver tissue are marked by a thin arrow. Bar A: 200 µm, bar C,D: 100 µm, bar B: 25 µm.*

The anti-apoptotic marker Bcl-2 was expressed to a higher extent in tumor than in normal hepatocytes (Figure 7A, B), whereas the apoptotic marker active caspase3 was strongly expressed in intrasplenic tumor tissue and even higher in intrahepatic nodules (Figure 7C, D). A summary of the different intrasplenic and intrahepatic staining results is given in Table 3. Together, these stainings show that the HuH6 cells in the intrahepatic tumors differed from those in the intrasplenic tumors by a lower BrdU incorporation, a lower CPS, cytokeratin7, and CD10 staining, and an increased cytokeratin18 and -19, active caspase3, and β-catenin staining.

Figure 7 (*see colour pages 151*) *BCL2 (A,B) and active Caspase3 (C,D) expression in HuH6 tumor in the spleen (A,C) and liver (B,D). Note BCL2-negative nodules in spleen. Tumor tissue in B is marked by an asterisk, host liver tissue by an arrow. Bar A: 200 µm, bar C,D: 100 µm, bar B: 50 µm.*

Table 3 *Immunostainings. A summary of the staining results of HuH6 cells and HuH6 tumor nodules in spleen (i.s.) and liver (i.h.) is given*

AB[a]	host species (AB type)	specificity	HuH6 Cells	HuH6 tumor i.s	i.h.
α-FP	mouse	human	++	+	+
BCL2	mouse (IgG1)	human	–	++	+/–
β-catenin	mouse	human	membranous	nuclear	nuclear
BrdU	mouse (MoAb)	–	nd	+	+/–
Caspase 3	mouse (MoAb)	human	+	++	+++
CD10	mouse	human	nd	+	+/– to –
CK19	mouse (IgG1)	human	+	++	+++
CK18	mouse (MoAb)	human	nd	++	+++
CK7	mouse (MoAb)	human	nd	+/–	–
c-MET	mouse (MoAb)	human, mouse	+/–	+	+
CPS	rabbit	human, mouse	+	heterogenous	+/–
GS	mouse (MoAb)	human, rat	+	+/–	+/–
OV-6	rabbit	human	+	heterogenous	++

[a] See text for abbreviations.

AB: antibody; nd: not determined; +++: very strong; ++: strong; +: weak; +/-: very weak; -: absent

Discussion

To study HB not only in cell culture, but also in a more physiologic environment, *in vivo* subcutaneous models with xenotransplanted HB have been established[10-15]. Easy access to measure tumor size and monitor experimental treatments are well-known advantages of such models[34,35]. In most cases, however, these subcutaneous tumors grow as encapsulated, poorly vascularized masses[8]. In the present study, we have established and characterized an intrahepatic xenograft model of human HB. Many of the phenotypic features of HB were retained. The development of the tumor could be monitored by following the increase in the circulating concentration of the human α-fetoprotein in tail-vein plasma. A prominent difference between HB cells *in vitro* and *in vivo* was the 5-fold lower growth rate *in vivo* than *in vitro.* Furthermore, the apparent spread by metastasis of HuH6 from the spleen to the liver was associated with the loss of some differentiation features. Finally, the intrahepatic HuH6 nodules differed from primary HB nodules by a deficient interaction with the surrounding liver or spleen tissue as evidenced by the absence of vessels. The take rate of HB and HCC cells in our model was relatively low and can probably be increased by using cells that were serially transplanted from mouse to mouse. It is, however, un-

likely that this modification would have altered the phenotypic characteristics of the intrahepatic HuH6 nodules.

Intriguing questions are why tumors such as HepT1 and HepG2 can loose their capacity to grow subcutaneously and intrahepatically, respectively, and especially why these tumor cells grow more easily subcutaneously than intrahepatically. Apparently, murine liver is a rather hostile environment for human hepatocytes, because human hepatocytes injected into the portal vein of immunodeficient mice completely disappeared from the liver of those mice with an apparent half life of ~8 hours, whereas the survival of such hepatocytes after transplantation to the subcutaneous or renal subcapsular space, was 20x and 50x longer, respectively[36]. Both the HuH6 and HepG2 tumors grew, nevertheless, much slower subcutaneously than *in vitro.* Furthermore, the intrahepatic HuH6 nodules did not contain blood vessels. The slower *in vivo* growth may, therefore, reflect environmental problems, like a deficient supply of growth factors and slow or absent vessel growth. The finding that the primary HepT1 tumor and its successful subcutaneous transplants expressed insulin-like growth-factor II, whereas these cells do not express this gene *in vitro*[37], may point towards such a regulatory mechanism. The association of HB growth *in vivo* with an active hepatocyte growth factor (HGF)-cMET axis and the expression of fibroblast-growth factor receptors also fits within this concept[17,36,38]. Activation of the HGF-cMET axis may have played a role in establishing HuH6 tumors in the spleen and liver, as c-MET expression was substantially upregulated in intrasplenic and intrahepatic HuH6 nodules (Figure 6C, D). Also, nuclear translocation of β-catenin was a remarkable feature of both intrasplenic and intrahepatic nodules when compared to the cultured HuH6 cells. These findings are in line with the proposed role of HGF/cMet in β-catenin stabilization[39].

The advantages of direct tumor cell delivery to the liver via the intrasplenic route were a better distribution of the cells within the liver, the possibility of using more cells, and the filtering role of the spleen in preventing thrombo-embolic processes by cell clumps. If transplanted primary human hepatocytes have a survival advantage over the murine host hepatocytes, they engraft and functionally integrate in an organized fashion into the hepatic architecture[40-42]. Since human fetal hepatoblasts resemble mature human hepatocytes in this respect[43], we had hypothesized that an intrahepatic HB model would behave similarly and be well vascularized. Our study clearly shows that HuH6 cells do not integrate into the hepatic architecture but, instead, form nodules without vessels surrounded by an inconspicuous, thin pseudocapsule. The resulting hypoxic condition may explain the high active caspase3 levels. The near absence of lymphocytes near the tumor capsule argues against a disturbed tumor growth due to an active immune response. We hypothesize that, instead, the difference in the behavior of primary hepatocytes and HB cells has to be ascribed to the colonization of the liver by metastasis rather than by direct delivery of the intrasplenically injected HB cells. Spread by metastasis is supported by the finding that, 6 weeks after intrasplenic injection, HuH6 cells still invade splenic vessels (Figure 3) and form thrombi in the portal vein (Figure 4). The presence of a large tumor nodule in a portal tract (Figure 5) further backs a model in which HB-cell aggregates become trapped in the smaller branches of the

portal vein. This localization and configuration appear to underscore that HB cells are not able to integrate into the hepatic architecture.

The colonization of the liver by metastasis of cells from the spleen raised the question to what extent the cells in the HuH6 nodules in the liver differed from the parent cells in the spleen. The intrahepatic hepatoblastoma nodules continue to express cytokeratin18, but not cytokeratin7, suggesting that, if anything, they represented a hepatocyte rather than a biliary lineage. It has been suggested that CK19+/7- cells harbor the adult hepatic stem cells[44]. They only weakly expressed α-fetoprotein and GS, sensitive markers for primary HB[45]. More important, perhaps, they only weakly expressed CPS (Figure 4), a hepatocyte-specific marker that only disappears from primary HB when they loose their epithelial phenotype (*Chapter V*) and CD10, a polarization marker for hepatocytes (Figure 6B) that is lost in metastatic HCC[46]. Our study has, therefore, shown that it is feasible to establish a realistic intrahepatic model for HB, but at the same time the study also revealed substantial phenotypic differences between the intrahepatic HB nodules that originated from the established cell line HuH6 and primary HB nodules. In primary HB nodules, the expression of αFP, GS, CPS, CK19 is high, but a substantial heterogeneity in staining exists between nodules in a single liver (*Chapter V*)[47,48], whereas the phenotype of the HuH6 tumors in the spleen or liver was remarkably homogeneous. In all likelihood, these differences can be traced to the marked environmental differences that exist between the *in vitro* cell culture environment to which the HuH6 cells have adapted during numerous passages and the liver *in situ*. For this reason, novel transplantable HB lines should be established by passing them through the liver rather than adapting them to an *in vitro* environment.

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References

1. Vos A. Primary liver tumours in children. *Eur J Surg Oncol* 1995; 21(1):101-5.
2. Perilongo G, Shafford EA. Liver tumours. *Eur J Cancer* 1999; 35(6):953-9.
3. Pritchard J, da Cunha A, Cornbleet MA, Carter CJ. Alpha feta (alpha FP) monitoring of response to adriamycin in hepatoblastoma. *J Pediatr Surg* 1982; 17:429-30.
4. von Schweinitz D, Hecker H, Schmidt-von-Arndt G, Harms D. Prognostic factors and staging systems in childhood hepatoblastoma. *Int J Cancer* 1997; 74(6):593-9.
5. Pritchard J, Brown J, Shafford E, Perilongo G, Brock P, Dicks-Mireaux C, et al. Cisplatin, doxorubicin, and delayed surgery for childhood hepatoblastoma: a successful approach--results of the first prospective study of the International Society of Pediatric Oncology. *J Clin Oncol* 2000; 18(22):3819-28.
6. Ortega JA, Douglass EC, Feusner JH, Reynolds M, Quinn JJ, Finegold MJ, et al. Randomized comparison of cisplatin/vincristine/fluorouracil and cisplatin/continuous infusion doxorubicin for treatment of pediatric hepatoblastoma: A report from the Children's Cancer Group and the Pediatric Oncology Group. *J Clin Oncol* 2000; 18(14):2665-75.
7. Schnater JM, Aronson DC, Plaschkes J, Perilongo G, Brown J, Otte JB, et al. Surgical view of the treatment of patients with hepatoblastoma: results from the first prospective trial of the International Society of Pediatric Oncology Liver Tumor Study Group. *Cancer* 2002; 94(4):1111-20.
8. Leveille-Webster CR, Arias IA. Establishment and serial quantification of intrahepatic xenografts of human hepatocellular carcinoma in severe combined immunodeficiency mice, and development of therapeutic strategies to overcome multidrug resistance. *Clin Cancer Res* 1996; 2:695-706.
9. Miyoshi E, Noda K, Ko JH, Ekuni A, Kitada T, Uozumi N, et al. Overexpression of alpha1-6 fucosyltransferase in hepatoma cells suppresses intrahepatic metastasis after splenic injection in athymic mice. *Cancer Res* 1999; 59:2237-43.
10. Hata Y, Uchino J, Sato K, Sasaki F, Une Y, Naito H, et al. Establishment of an experimental model of human hepatoblastoma. *Cancer* 1982; 50:97-101.
11. Suzuki T, Watanabe K, Hirota M, Iwafuchi M, Ono T, Aoyagi Y, Ito S. Establishment of a human hepatoblastoma model in athymic nude mice. Producibility of fatty acid-binding protein, alpha-fetoprotein, and alpha 1-acid glycoprotein. *Acta Pathol Jpn* 1992; 42:255-61.
12. Desdouets C, Fabre M, Gauthier F, Brechot C, Sobczak-Thepot J. Proliferation and differentiation of a human hepatoblastoma transplanted in the Nude mouse. *J Hepatol* 1995; 23:569-77.
13. Fuchs J, Schmidt D, Pietsch T, Miller K, von Schweinitz D. Successful transplantation of human hepatoblastoma into immunodeficient mice. *J Pediatr Surg* 1996; 31:1241-6.
14. Fuchs J, Wenderoth M, von Schweinitz D, Haindl J, Leuschner I. Comparative activity of cisplatin, ifosfamide, doxorubicin, carboplatin, and etoposide in heterotransplanted hepatoblastoma. *Cancer* 1998; 83:2400-7.
15. Fuchs J, Gratz KF, Habild G, Gielow P, von Schweinitz D, Baum RP. Immunoscintigraphy of xenotransplanted hepatoblastoma with iodine 131-labeled anti-alpha-fetoprotein monoclonal antibody. *J Pediatr Surg* 1999; 34:1378-84.
16. Pietsch T, Fonatsch C, Albrecht S, Maschek H, Wolf HK, von Schweinitz D. Characterization of the continuous cell line HepT1 derived from a human hepatoblastoma. *Lab Invest* 1996; 74:809-18.
17. von Schweinitz D, Faundez A, Teichmann B, Birnbaum T, Koch A, Hecker H, et al. Hepatocyte growth-factor-scatter factor can stimulate post-operative tumor-cell proliferation in childhood hepatoblastoma. *Int J Cancer* 2000; 85:151-9.
18. Doi I. Establishment of a cell line and its clonal sublines from a patient with hepatoblastoma. *Gann* 1976; 67:1-10.
19. Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* 1979; 282:615-6.
20. Flanagan SP. 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet Res* 1966; 8:295-309.
21. Segre JA, Nemhauser JL, Taylor BA, Nadeau JH, Lander ES. Posistional cloning of the nude locus: genetic, physical, and transcription maps of the region and mutations in the mouse and rat. *Genomics* 1995; 28: 549-59.

22. Fogh J, Fogh JM, Orfeo T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 1977; 59:221-6.
23. Carlsson G, Gullberg B, Hafstrom L. Estimation of liver tumor volume using different formulas – an experimental study in rats. *J Cancer Res Clin Oncol* 1983; 105:20-3.
24. Charles R, De Graaf A, Lamers WH, Moorman AF. Control of the changes in rat-liver carbamoyl-phosphate synthase (ammonia) protein levels during ontogenesis: evidence for a perinatal change in immunoreactivity of the enzyme. *Mech Ageing Dev* 1983; 22:193-203.
25. Dunsford HA, Karnasuta C, Hunt JM, Sell S. Different lineages of chemically induced hepatocellular carcinoma in rats defined by monoclonal antibodies. *Cancer Res* 1989; 49:4894-900.
26. Duan WR, Garner DS, Williams SD, Funckes-Shippy CL, Spath IS, Blomme EA. Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. *J Pathol* 2003; 199:221-8.
27. Niwa Y, Matsumura M, Shiratori Y, Imamura M, Kato N, Shiina S, et al. Quantitation of alpha-fetoprotein and albumin messenger RNA in human hepatocellular carcinoma. *Hepatology* 1996; 23:1384-92.
28. Gaasbeek Janzen JW, Gebhardt R, ten Voorde GH, Lamers WH, Charles R, Moorman AF. Heterogeneous distribution of glutamine synthetase during rat liver development. *J Histochem Cytochem* 1987; 35:49-54.
29. Gebhardt R, Mecke D. Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cells in situ and in primary culture. *Embo J* 1983; 2:567-70.
30. Ruck P, Xiao JC, Kaiserling E. Small epithelial cells and the histogenesis of hepatoblastoma. Electron microscopic, immunoelectron microscopic, and immunohistochemical findings. *Am J Pathol* 1996; 148:321-9.
31. Pezzella F, Turley H, Kuzu I, Tungekar MF, Dunnill MS, Pierce CB, et al. bcl-2 protein in non-small-cell lung carcinoma. *N Engl J Med* 1993; 329:690-4.
32. Ishak KG, Goodman ZD, Stocker JT. Hepatoblastoma. Tumors of the liver and intrahepatic bile ducts; Armed Forces Institute of Pathology: Washington DC, 2001; 159-84.
33. Matsuura S, Aishima S, Taguchi K, Asayama Y, Terashi T, Honda H, Tsuneyoshi M. 'Scirrhous' type hepatocellular carcinomas: a special reference to expression of cytokeratin 7 and hepatocyte paraffin 1. *Histopathology* 2005; 47:382-90.
34. Ain JF, Gouillat C, Bertrand S, Fourel I, Guillaud M, Saguier G, Trepo C. Human hepatocellular carcinoma transplanted in nude mice: a relevant experimental model to assess tumoral destruction by alcoholization. *J Surg Res* 1994; 57:366-72.
35. Labonte P, Kadhim S, Bowlin T, Mounir S. Inhibition of tumor growth with doxorubicin in a new orthotopically implanted human hepatocellular carcinoma model. *Hepatol Res* 2000; 18:72-85.
36. Ohashi K, Marion PL, Nakai H, Meuse L, Cullen JM, Bordier BB, et al. Sustained survival of human hepatocytes in mice: A model for in vivo infection with human hepatitis B and hepatitis delta viruses. *Nat Med* 2000; 6:327-31.
37. Eriksson T, Frisk T, Gray SG, von Schweinitz D, Pietsch T, Larsson C, et al. Methylation changes in the human IGF2 p3 promoter parallel IGF2 expression in the primary tumor, established cell line, and xenograft of a human hepatoblastoma. *Exp Cell Res* 2001; 270:88-95.
38. Asada N, Tanaka Y, Hayashido Y, Toratani S, Kan M, Kitamoto M, et al. Expression of fibroblast growth factor receptor genes in human hepatoma-derived cell lines. *In Vitro Cell Dev Biol Anim* 2003; 39:321-8.
39. Monga SP, Mars WM, Pediaditakis P, Bell A, Mule K, Bowen WC, et al. Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. *Cancer Res* 2002; 62:2064-71.
40. Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, Sommer G, et al. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. *Hepatology* 2001; 33:981-8.
41. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004; 165:901-12.
42. Meuleman P, Libbrecht L, De Vos R, de Hemptinne B, Gevaert K, Vandekerckhove J, et al. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology* 2005; 41:847-56.
43. Mahieu-Caputo D, Allain JE, Branger J, Coulomb A, Delgado JP, Andreoletti M, et al. Repopulation of athymic mouse liver by cryopreserved early human fetal hepatoblasts. *Hum Gene Ther* 2004; 15:1219-28.

44. Paku S, Dezso K, Kopper L, Nagy P. Immunohistochemical analysis of cytokeratin 7 expression in resting and proliferating biliary structures of rat liver. *Hepatology* 2005; 42:863-70.
45. Schnater JM, Kohler SE, Lamers WH, von Schweinitz D, Aronson DC. Where do we stand with hepatoblastoma? A review. *Cancer* 2003; 98:668-78.
46. Saad RS, Luckasevic TM, Noga CM, Johnson DR, Silverman JF, Liu YL. Diagnostic value of HepPar1, pCEA, CD10, and CD34 expression in separating hepatocellular carcinoma from metastatic carcinoma in fine-needle aspiration cytology. *Diagn Cytopathol* 2004; 30:1-6.
47. Ruck P, Xiao JC, Pietsch T, von Schweinitz D, Kaiserling E. Hepatic stem-like cells in hepatoblastoma: expression of cytokeratin 7, albumin and oval cell associated antigens detected by OV-1 and OV-6. *Histopatology* 1997; 31:324-9.
48. Zimmerman A. Hepatoblastoma with cholangioblastic features ('cholangioblastic hepatoblastoma') and other liver tumors with bimodal differentiation in young patients. *Med Pediatr Oncol* 2002; 39:487-91.

'Finally all the results together,
so time for the appendices
and summary

Chapter 7

Appendix I

Appendix I

Acknowledgment

We would like to like to emphasize that the study presented in *Chapter III and IV* could only be conducted with the participation of the following centres: *Argentina:* Buenos Aires, Italian Hospital of Buenos Aires, *Australia:* Adelaide, Adelaide Children's Hospital; Brisbane, Royal Children's Hospital; Melbourne, Royal Children's Hospital; Paramatta, The New Children's Hospital; Westmead, Westmead Hospital, *Belgium:* Brussels, Cliniques Universitaires Saint-Luc; Brussels, Hopital Universitaire des Enfants; Gent, University Hospital/ Kliniek voor Kinderziekten; Leuven, University Hospital Gasthuisburg; Montegnee, Clinique de Montegnee, *Brazil:* Sao Paulo, AC Camargo Hospital; Sao Paulo, Amico Hospital; Sao Paulo, Centro Infantil; Sao Paulo, Hospital Servidor Publico Estadual; Sao Paulo, Santa Casa, *Croatia:* Zagreb, Children's Clinic Salata, *Czechoslovakia:* Banska Bystrica, Paediatric Oncological Centre/ Regional Hospital; Prague, Clinic of Children Oncolgy, *Denmark:* Copenhagen, University Hospital; Odense, Odense University Hospital, *Egypt:* Alexandria, University of Alexandria, *Finland:* Helsinki, Children's Hospital, *France:* Lille, Centre Oscar Lambret; Lyon, Centre Leon Berard; Nancy, Hopital d'Enfants; Paris, Institut Curie, *Germany:* Tubingen, University of Tubingen/ Eberhard Karls Universitat, *Greece:* Athens, Children's Hospital; Thessaloniki, Ippokation Hospital, *Hungary:* Budapest, Semmelweis University Medical School, Miskolc, Miskolc Medical School, *Ireland:* Dublin, Our Lady's Hospital for Sick Children, *Israel:* Haifa, Rambam Medical Centre, *Italy:* Bari, Policlinico Universita Bari; Genova, Giannina Gaslini Children's Hospital; Padova, Chirurgica Pediatrica; Torino, Ospedale Regina Margherita, *Japan:* Saporro, Sapporo National Hospital, *Malaysia:* Kelantan, Hospital Universiti Sains Malaysia, *Netherlands:* Amsterdam, Emma Kinder Ziekenhuis/ Academic Medical Center; Amsterdam, Free University; Leiden, University Hospital of Leiden; Nijmegen, University Hospital Nijmegen, *New Zealand:* Auckland, Starship Children's Hospital; Wellington South, Wellington School of Medicine, *Northern Ireland:* Belfast, Royal Hospital for Sick Children, *Norway:*Bergen, University Hospital; Oslo, Rikshospitalet, *Poland:* Szczecin, Pomeranian Medical Academy; Warsaw, Research Institute of Mother & Child; Wroclaw, Medical Academy, *Portugal:* Porto, Hospital St Antonio, *Slovenia:* Ljubljana, University Paediatric Hospital, *South Africa:* Cape town, Red Cross Children's Hospital; Johannesburg, Baragwanath Hospital; Pretoria, Kalatonge Hospital; Tygerberg, Tygerberg Hospital, *Spain:* Barcelona, Hospital Infantil Valle Hebron; Bilbao, Hospital Infantil de Cruces; Malaga, Hospital Materno-Infantil; Valencia, Hopital "La Fe", *Sweden:* Goteborg, University of Goteborg; Lund, University Hospital; Stockholm, Karolinska Hospital, *Switzerland:* Bern, Universitäts-Kinderklinik; Lausanne, University Hospital (CHUV); Zurich, Children's University Clinic, *Taiwan R.O.C.*: Taipei, National Taiwan University Hospital, *Turkey:* Ankara, Hacattepe University, *United Kingdom:* Aberdeen, Royal Aberdeen Children's Hospital; Birmingham, The Children's Hospital; Bristol, Royal Hospital for Sick Children; Edinburgh, Royal Hospital for Sick Children; Glasgow, Royal Hospital for Sick Children; Leeds, St. James' University Hospital; Leicester, Leicester Royal Infirmary; Liverpool, Royal Liverpool Children's Hospital; London,

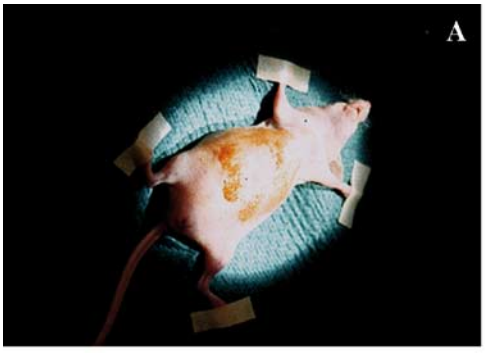
Hospital for Sick Children; London, King's College Hospital; London, Middlesex Hospital; London, Royal Marsden Hospital; London, St. Bartholomew's Hospital; Manchester, Royal Manchester Children's Hospital; Newcastle, Royal Victoria Infirmary; Nottingham, Queen's Medical Centre; Oxford, John Radcliff Hospital; Sheffield, Children's Hospital; Southampton, Southampton General Hospital, *Uruguay:* Montevideo, Hospital Pereira Rossell.

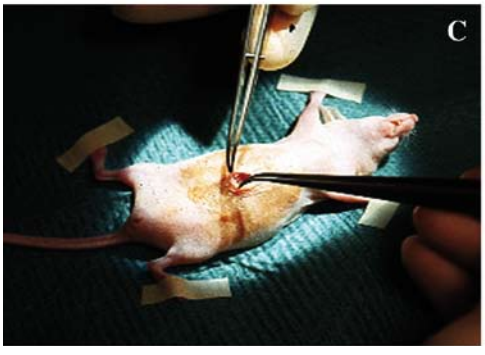
Appendix II

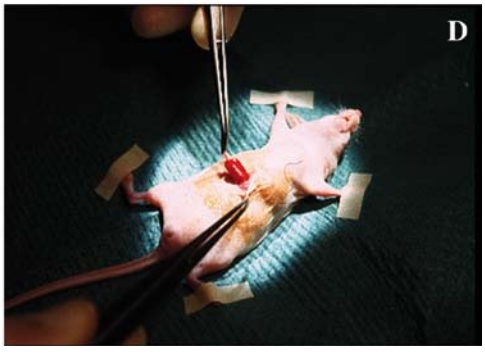
Appendix II

Surgical procedure for intrahepatic tumor model

Mice were anesthestized with: Fentanylcitrate 0.315 mg/ml, Fluanison 10 mg/ml, Midazolam 5 mg/ml, and H_2O (1:1:1:2) (7 ml/kg body weight), and operated on a 43° C degrees (pre)warmed operation table. Antisepsis was performed using Betadine® (**A**). A small median laparotomy of 1 cm was performed. Skin incision (**B**), linea alba incision for abdominal cavity access (**C**). Extra-abdominal fixation of the spleen (**D**). Injection of 200µl tumor cell suspension into the lower pole of the spleen with a 30 G needle (**E**). Coagulation with surgical sponge (K-sponge® for micro-surgery, Kalena products, Inc.) (**F**). The spleen is returned into the abdominal cavity (**G**), and 300 µl of 0.9% sodium chloride is injected intraperitoneally for fluid substitution (**H**). The abdomen is closed in 2 layers: muscle-layers with silk sutures 6x0 (Silkam, Polymed®) (**I, J**), skin with 7.5x1.75 mm wound-clips ('Michel', Eisenhut-Vet AG®) (**K, L**). Postoperative, mice were kept warm under a 150 W infrared lamp (Osram®, Siccatherm) and returned to their cages when fully awake.





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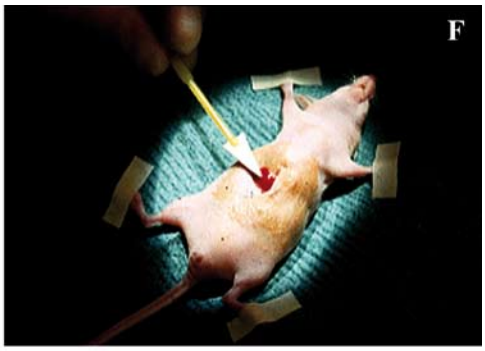
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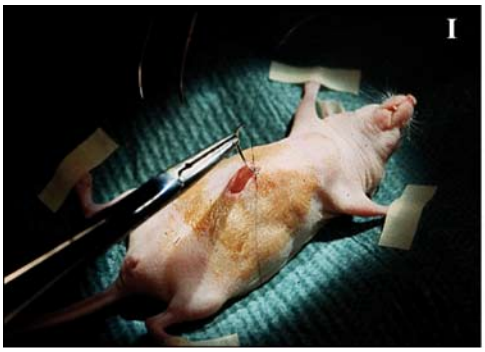
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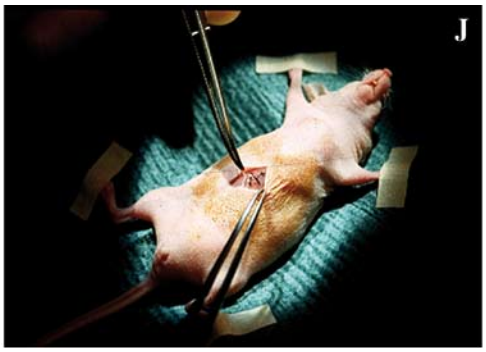

J


K


L

Samenvatting
Summary

Samenvatting

Het hepatoblastoom (HB) is een zeldzame maligniteit van de lever dat meestal wordt gezien bij kinderen jonger dan 3 jaar. *Hoofdstuk I* geeft een overzicht van de huidige klinische en biologische eigenschappen van deze intrigerende tumor. Er werd aangetoond dat met name de combinatie van chemotherapie en chirurgie debet is aan de enorme verbetering van de overleving gedurende de afgelopen 3 decennia. Grote internationale trials hebben geleid tot een hedendaagse genezingstendens van ongeveer 75%, alhoewel extrahepatische tumoruitbreiding, multifocaliteit van de tumor en metastasering op afstand geassocieerd zijn met een slechtere prognose. Cytogenetische en moleculair biologische studies onthullen de fenotypische eigenschappen, cytogenetische veranderingen en de mogelijke rol van cytokines, β-catenine en het Wnt signaal pad van deze kwaadaardige tumor in meer detail. Desalniettemin hebben zij tot op de dag van vandaag nog niet geleid tot de ontdekking van betrouwbare prognostische factoren.

In *Hoofdstuk II* werden de resultaten van preoperatieve biopsie en chirurgie bestudeerd in die patiënten die zich de afgelopen 24 jaar in het Kinderchirurgisch Centrum Amsterdam presenteerden met een primaire levertumor. Wij toonden aan dat, met uitzondering van het hepatocellulair carcinoom (HCC), de behandelingsresultaten van verschillende levertumoren op de kinderleeftijd door middel van chirurgische resectie en (neo)adjuvante chemotherapie overeenkomen met de resultaten van grote internationale studies. Bovendien werd aangetoond dat de bestaande diagnostische valkuilen tussen de verschillende levertumoren onderling een diagnostische biopsie rechtvaardigen en zelfs noodzakelijk maken. Zeker als men de veiligheid van de huidige biopsietechnieken en de goede histologische voorspellende waarde in ogenschouw neemt.

De International Society of Pediatric Oncology begon in 1990 de eerste prospectieve trial (SIOPEL-1) met de intentie om alle kinderen met een HB te behandelen met preoperatieve chemotherapie en uitgestelde chirurgische resectie. Zij ontwikkelden een nieuw stagiëring systeem puur en alleen gebaseerd op beeldvormende technieken, te weten het PRE-Treatment EXTent of disease systeem (PRETEXT). Dit systeem werd gebruikt om de tumor respons na verschillende kuren chemotherapie te evalueren en te analyseren op welk tijdstip chirurgische resectie kon worden verricht. In *Hoofdstuk III* analyseerden wij van de 154 geïncludeerde kinderen in de SIOPEL-1 studie de 128 kinderen die tussen 1990 en 1994 een chirurgische resectie van hun HB ondergingen. Wij bevestigden de veiligheid van een biopsie en adviseerden deze standaard toe te passen. Verder werd aangetoond dat de resectie van de tumor 'eenvoudiger' wordt door de preoperatieve chemotherapie en dat een re-resectie van het tumor positieve resectievlak niet noodzakelijk is daar de resultaten van postoperatieve chemotherapie bij een tumor positief resectievlak goed waren. Resectie van longmetastasen kan curatief zijn als er locale controle van de primaire tumor bestaat, alhoewel de resultaten wel aantoonden dat de prognose van de patiënt slechter is. De chirurgische morbiditeit en mortaliteit waren in deze grote multicenter studie niet hoger, maar belangrijker, de trial toonde aan dat ook de landen met een mindere economische status effectief kunnen bijdragen aan dit soort studies.

In *Hoofdstuk IV* werd de accuratesse en de interobserver overeenkomst van het nieuwe PRETEXT systeem gebruikt in de SIOPEL-1 studie geanalyseerd. Tevens werd er een vergelijk gemaakt tussen de voorspellende waarde in relatie tot overleving van het PRETEXT systeem, het CCSG/POG stagiëringssysteem en het conventionele TNM systeem voor levercarcinoom (dat zijn de 3 huidig gebruikte stagiëringssystemen voor HB). Wij toonden aan dat het PRETEXT systeem: (1) een matige accuratesse vertoonde met een tendens om patiënten te 'overstadiëren'; (2) een goede interobserver overeenkomst vertoont (= reproduceerbaarheid); (3) een superieure voorspellende waarde voor overleving heeft in vergelijk tot de andere stagiëringssystemen; (4) de mogelijkheid biedt om het effect van de preoperative behandeling te beoordelen; en (5) ook kan worden toegepast op patiënten die niet worden geopereerd. Om de resultaten van verschillende studies beter te kunnen vergelijken, werd er geadviseerd om alle patiënten met een HB die geïncludeerd worden in trials ook te stagiëren volgens het PRETEXT systeem.

De epidemiologische data toonden aan dat ongeveer een kwart van de patiënten nog steeds overlijdt als gevolg van hun ziekte en de klinische data maakte het mogelijk om risicogroepen te identificeren. Om de levensverwachting van de groep kinderen met deze infauste prognose te verbeteren is het noodzakelijk om de tumor in meer detail te bestuderen en andere behandelingsstrategieën te testen. Om meer inzicht in de phenotypische eigenschappen van HB te krijgen bestudeerden wij in *Hoofdstuk V* de expressiepatronen van glutamine synthetase (GS) (wat is geassocieerd met activatie van β-catenine (opgereguleerd in HB)), carbamoyl fosfaat synthetase (CPS) (als marker voor de dedifferentiatie van hepatocyten), cytokeratine7 (CK7) en cytokeratine19 (CK19) (beide mogelijk leverstamcel markers) in 19 patiënten met een HB. Wij stelden voor om de groeipatroon eigenschappen (expansief vs. infiltratief), de grootte van phenotypisch homogene tumorhaarden (als afspiegeling van de mate van differentiatie), het vascularisatiepatroon en de zonatie van genexpressie binnen de tumorhaarden als nieuwe prognostische factoren te onderzoeken om de biologische eigenschappen van HB te doorgronden. Tot slot, om de mogelijkheid te hebben alternatieve behandelingsstrategieën voor het HB in een meer fysiologische omgeving te karakteriseren en de prognose van de 25% van de kinderen met een infaust HB mogelijk in de toekomst te verbeteren ontwikkelden wij in *Hoofdstuk VI* een orthotopisch humaan HB model in. Wij toonden aan dat het mogelijk was om naast een subcutaan model tevens een intrahepatisch HB model in naakte muizen te ontwikkelen door middel van intrasplenische injectie van tumorcellen. Tumor groei kon door middel van de expressie van de oplosbare tumor marker α-fetoprotein worden gedetecteerd en gecontroleerd in staartvene serum. De levertumoren ontwikkelden zich als metastase vanuit de milttumoren. Opvallend was het verlies van sommige differentiatie kenmerken van de intraheptische tumoren in vergelijking met de intrasplenische tumoren. Metastasen op afstand werden niet aangetoond en de efficiëntie van het model kan mogelijkerwijze worden verhoogd door de nieuwe tranplanteerbare HB cellijnen via de lever te laten passeren. Desalnietemin, kunnen beide modellen worden gebruikt om alternatieve en experimentele therapeutische strategieën te testen, zoals adenovirale suïcide gen therapie, nieuwe farmaca en nieuwe chemotherapeutische protocollen.

Summary

Hepatoblastoma (HB) is an uncommon liver malignancy that is seen mostly in children younger than 3 years. *Chapter I* presents an overview of the currently known clinical and biological characteristics of this intriguing tumor. It was shown that the dramatic increase in survival that has been observed in the last 3 decades, is due mainly to the combination of chemotherapy and surgery. Large international trials has led to a current cure rate of approximately 75% of children with HB, although extrahepatic tumor extention, multifocality of the tumor, and metastatic spread are associated with a poor prognosis. Cellular-biologic and molecular-biologic studies are revealing the phenotypic features, cytogenetic alterations, and possible role of cytokines, β-catenin and the Wnt signaling pathway of this malignant tumor in more detail; however, to date, they have not led to the discovery of reliable prognostic factors.

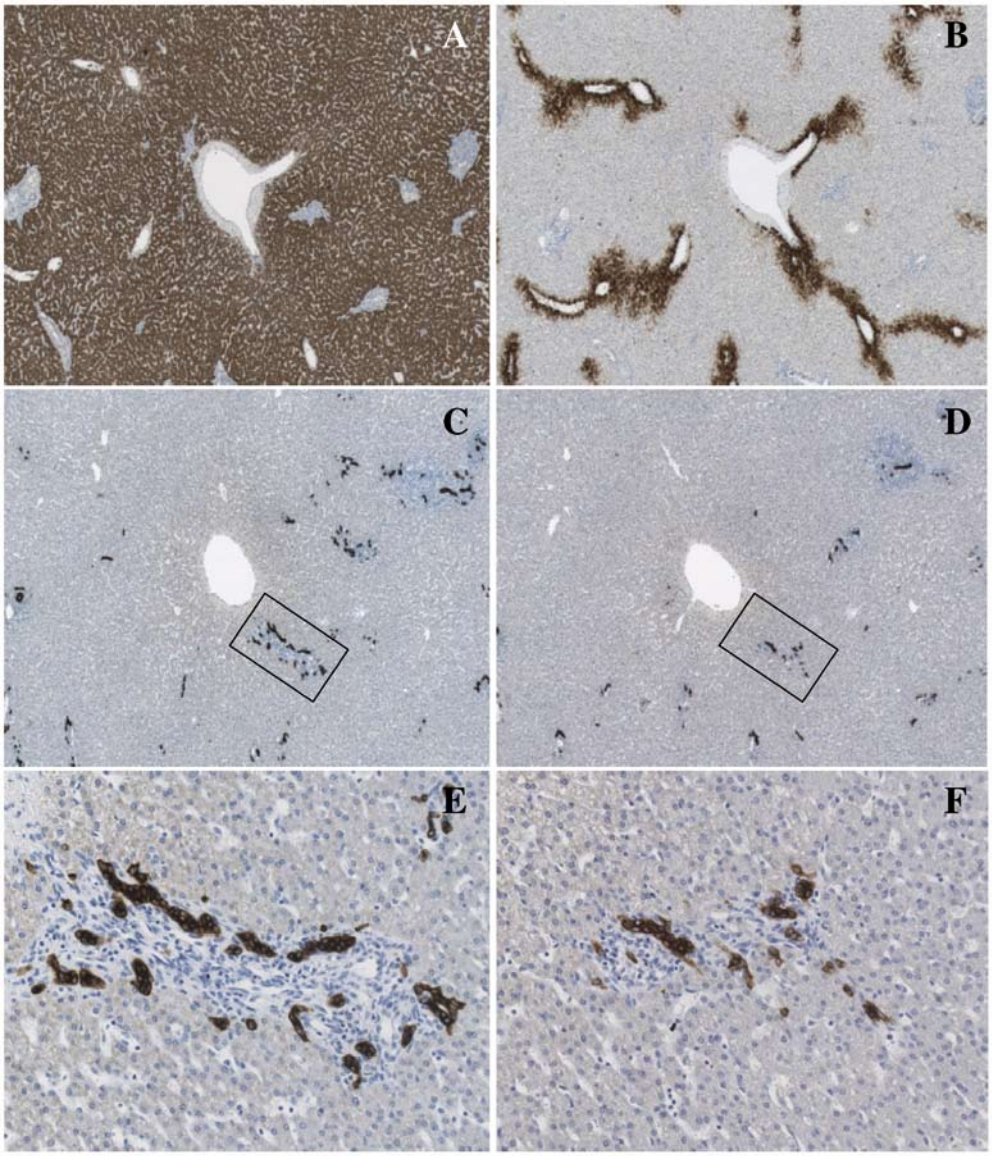
In *Chapter II* we studied our 24 years of pre-treatment biopsy and surgical experience in the Pediatric Surgical Center of Amsterdam for those patients presenting with a primary liver tumor. We showed that in our center the treatment results of various pediatric liver tumors with surgical resection and (neo) adjuvant chemotherapy are comparable with those of larger international series except for hepatocellular carcinoma (HCC). Furthermore, the existing diagnostic pitfalls in the differentiation between the various liver malignancies justify and necessitate the use of a diagnostic biopsy. Especially if one considers the safety of the current techniques and the good histological predictive value.

In 1990 the International Society of Pediatric Oncology launched the first prospective trial (SIOPEL-1) with the intention to treat all children with a HB with preoperative chemotherapy and delayed surgical resection. They developed a new staging system solely based on imaging techniques called the PRE-Treatment EXTent of disease system (PRETEXT). This system was used to evaluate the tumor response after different courses of chemotherapy and to analyze at what time point surgical resection could be performed. In *Chapter III*, we analyzed the 128 out of the 154 children included in the SIOPEL-1 study who underwent a surgical resection of their HB between 1990 and 1994. We confirmed the safety of a biopsy and recommend it to perform it routinely. It was also shown that preoperative chemotherapy seems to make tumor resection easier and that reresection of a positive resection margin does not necessarily have to be performed, because postoperative chemotherapy showed good results. If local control of the primary tumor exists, resection of lung metastases can be curative; however, results showed that the patient's prognosis was worse. Surgical morbidity or mortality rates were not necessarily higher in large multicenter studies, but more importantly, countries of lesser economic status also can contribute effectively to these trials.

In *Chapter IV,* the accuracy and interobserver agreement of the new PRETEXT staging system used in the SIOPEL-1 was analyzed. In addition, a comparison was made between the predictive impact of the PRETEXT staging system, the CCSG/POG staging system, and the conventional TNM system for (adult) liver carcinomas (i.e. the 3 currently used staging systems for HB) with

regard to overall survival. We showed that the PRETEXT system: (1) has moderate accuracy with a tendency to 'overstage' patients; (2) shows good interobserver agreement (= reproducibility); (3) shows superior predictive value for survival compared to the other staging systems; (4) offers the opportunity to monitor the effect of preoperative therapy; and, (5) can also be applied in patients who are not operated. It was therefore recommended to stage all HB patients included in trials according to PRETEXT, to make results from different trials more accurately comparable.
The epidemiological data showed that approximately a quarter of the patients still die as a result of their disease and risk groups could be identified on clinical data. To improve the outcome in the group of children with this infaust prognosis, the tumor must be analyzed in a more detailed manner and other treatment strategies has to be investigated. To gain insight in the phenotypic characteristics of HB we studied the expression patterns of glutamine synthetase (GS) (which is associated with activation of β-catenin (up-regulated in HB)), carbamoyl-phosphate synthetase (CPS) (as marker for hepatocyte dedifferentiation), cytokeratin7 (CK7), and cytokeratin19 (CK19) in 19 patients with a HB in *Chapter V*. We proposed to study the growth pattern (expansive vs. infiltrative), the size of phenotypically homogeneous nodules (reflecting the rate of dedifferentiation), the vascularization pattern and the zonation of gene expression in the nodules as new prognostic factors for understanding the biological characteristics of HB in more detail. Finally, to offer the opportunity to characterize and test alternative treatment strategies in HB in a more physiologic environment and thereby trying to improve the prognosis of the 25% of the children with an infaust HB we developed an orthoptic human HB tumor model in *Chapter VI*. We showed that it was possible to establish beside a subcutaneous, also an intrahepatic HB model in nude mice by means of intrasplenic tumor cell injection. Tumor growth could be detected and monitored by the expression of the soluble tumor marker α-fetoprotein in tail vein serum. Engrafting of the tumor in the liver occurred by metastatic spread from the spleen. Remarkable was the loss of some of the differentiation featured of the intrahepatic nodules. Distant metastases were not found and the efficiency of the model might be improved by passing the novel transplantable HB cell lines through the liver. However, both models can be used to test alternative and experimental therapeutic strategies like adenoviral suicide gene therapy, novel agents, and new chemotherapeutic protocols.

Colour images



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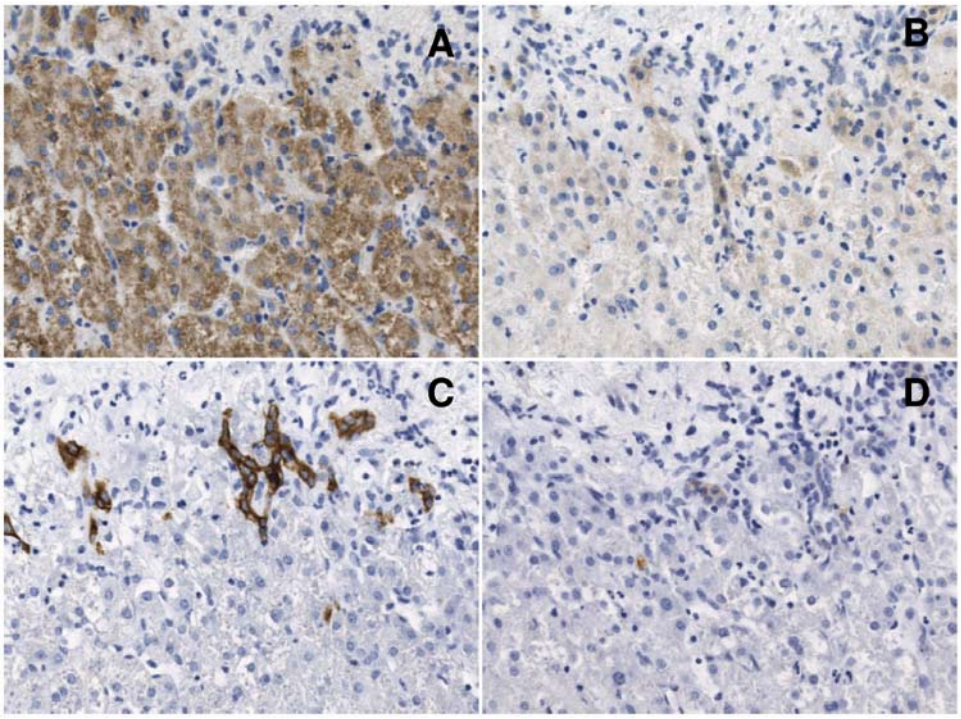
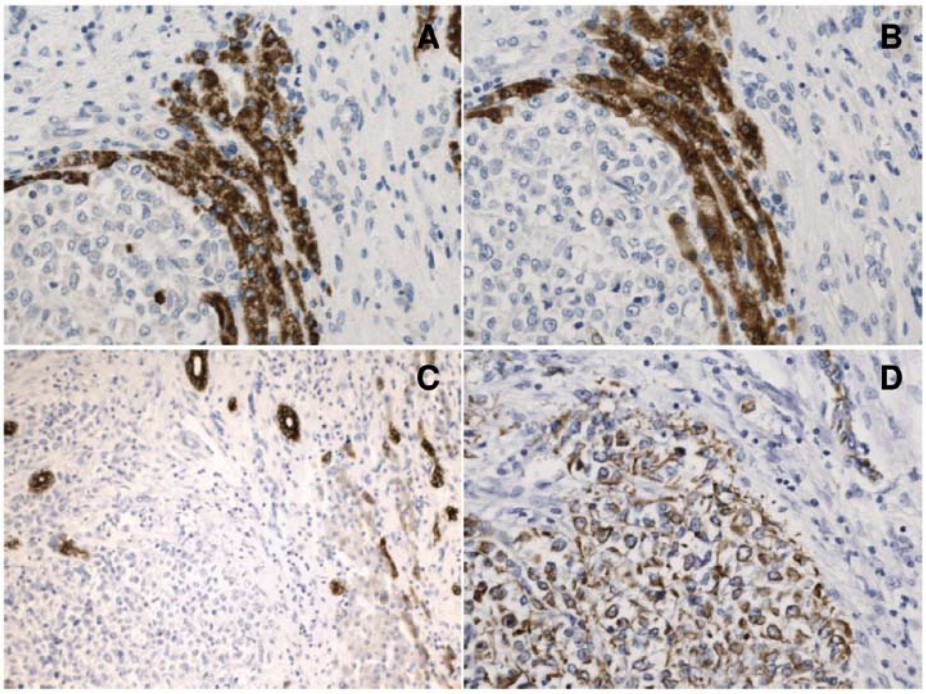


Chapter 5 **Figure 2**



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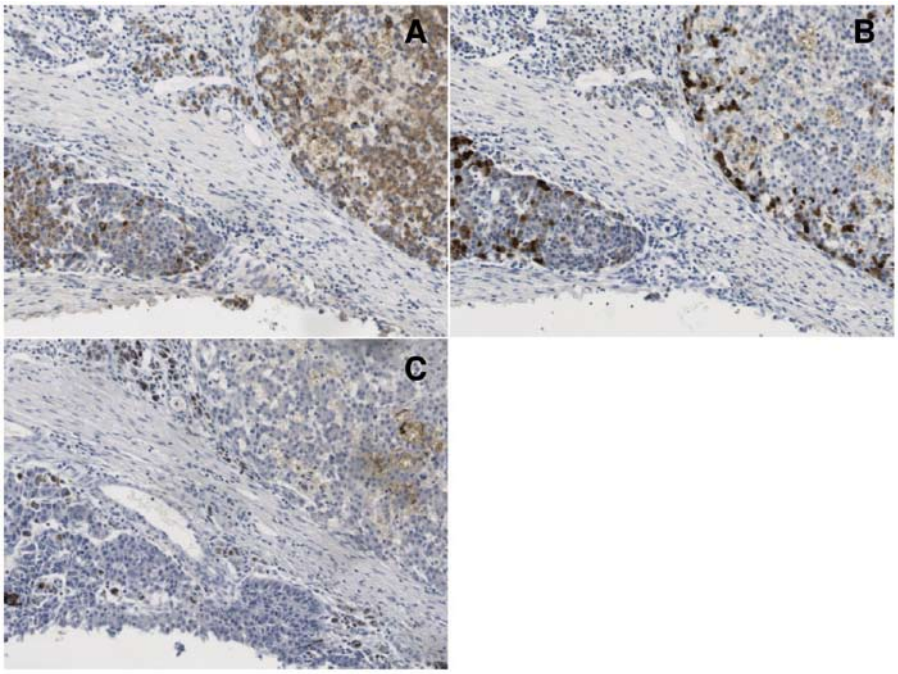

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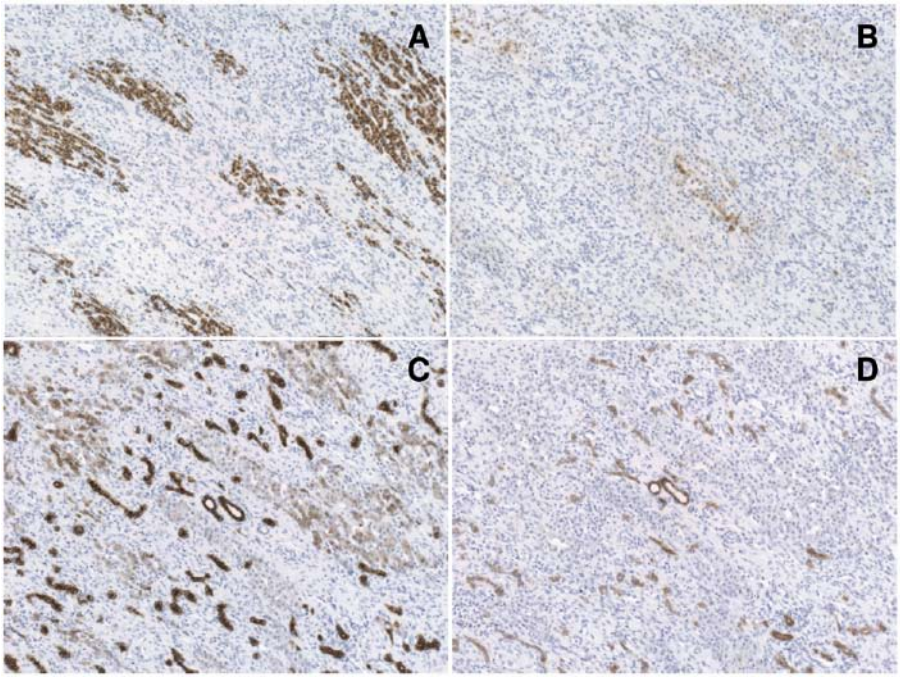

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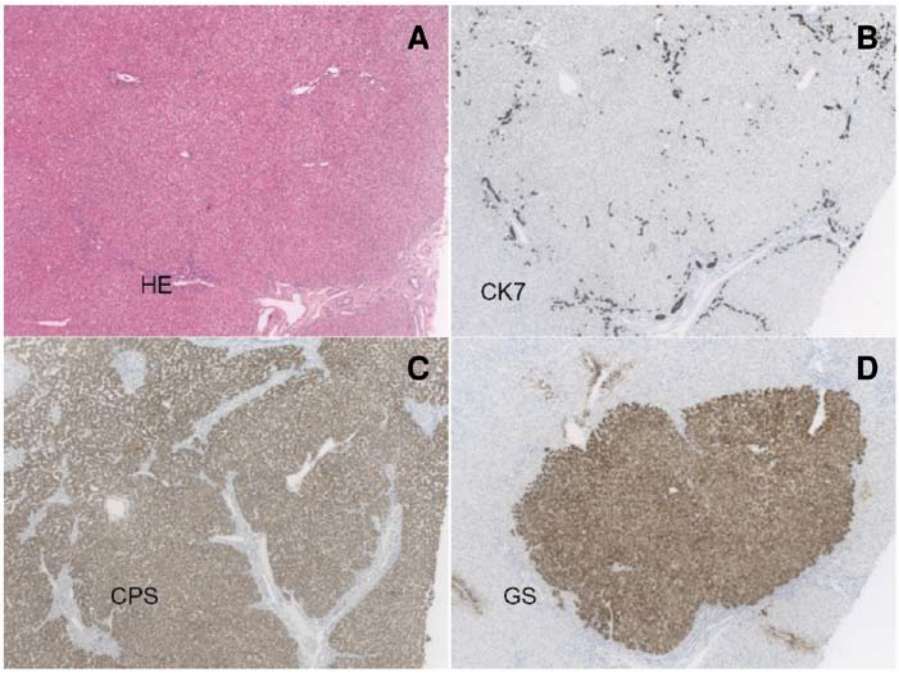

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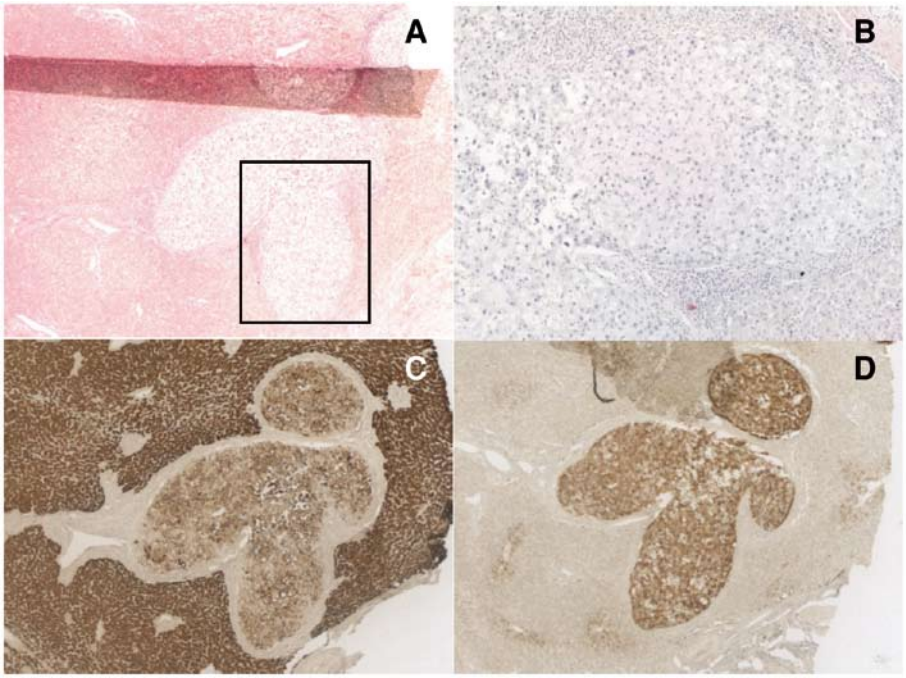

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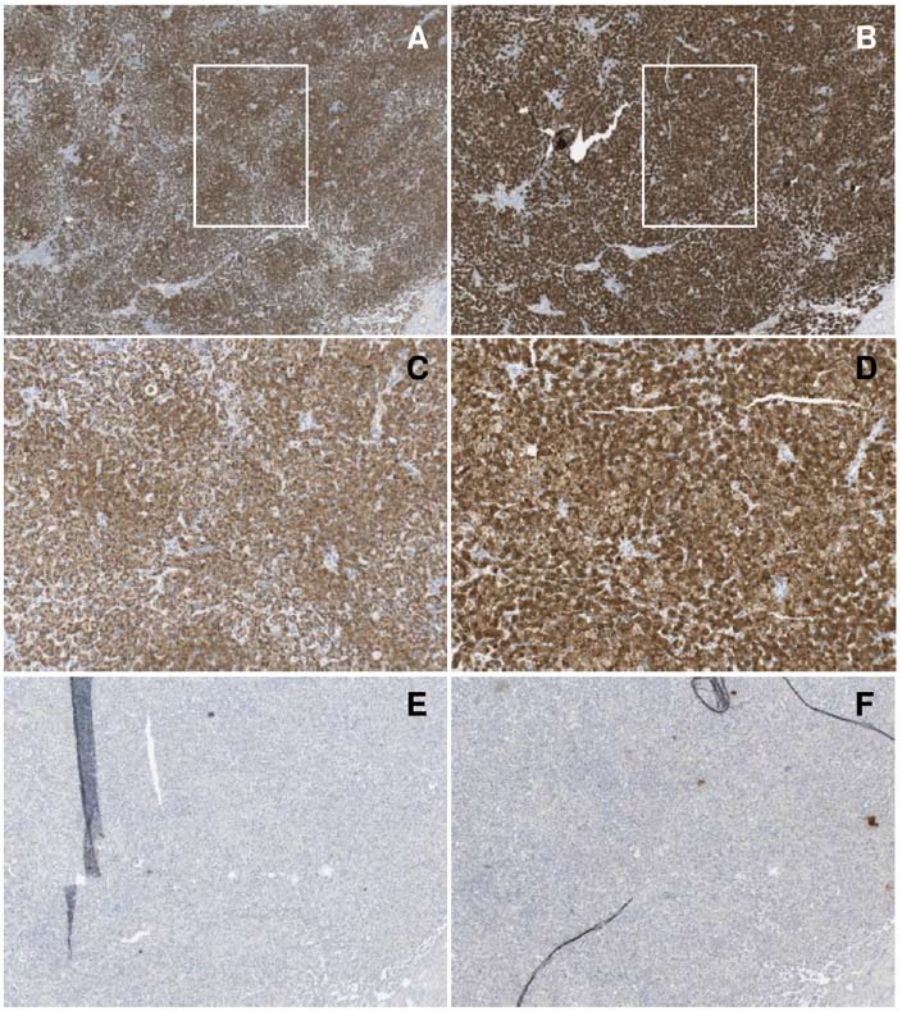

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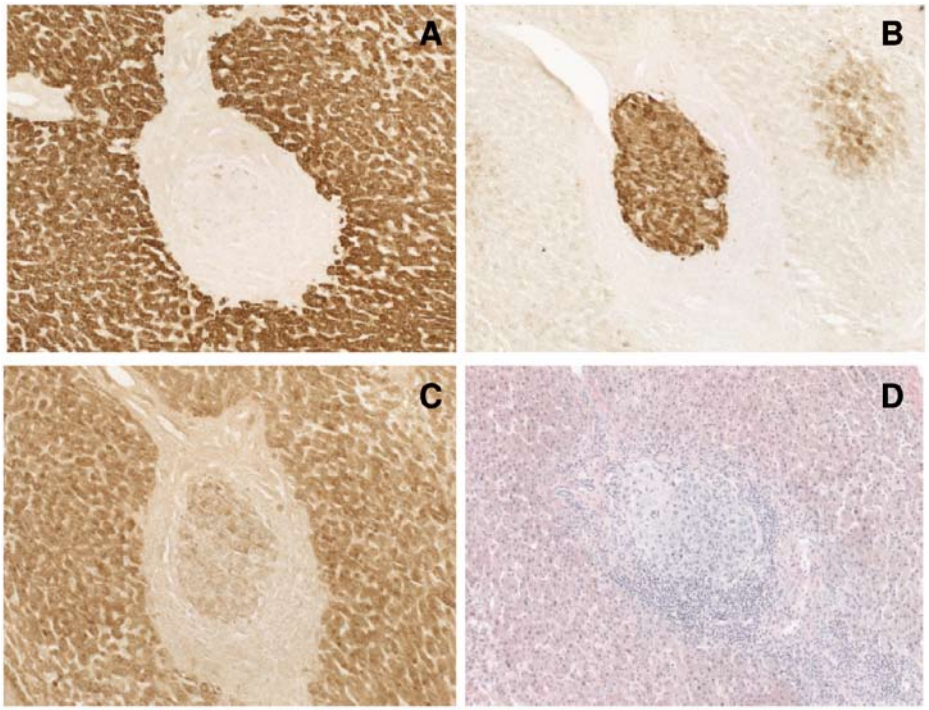

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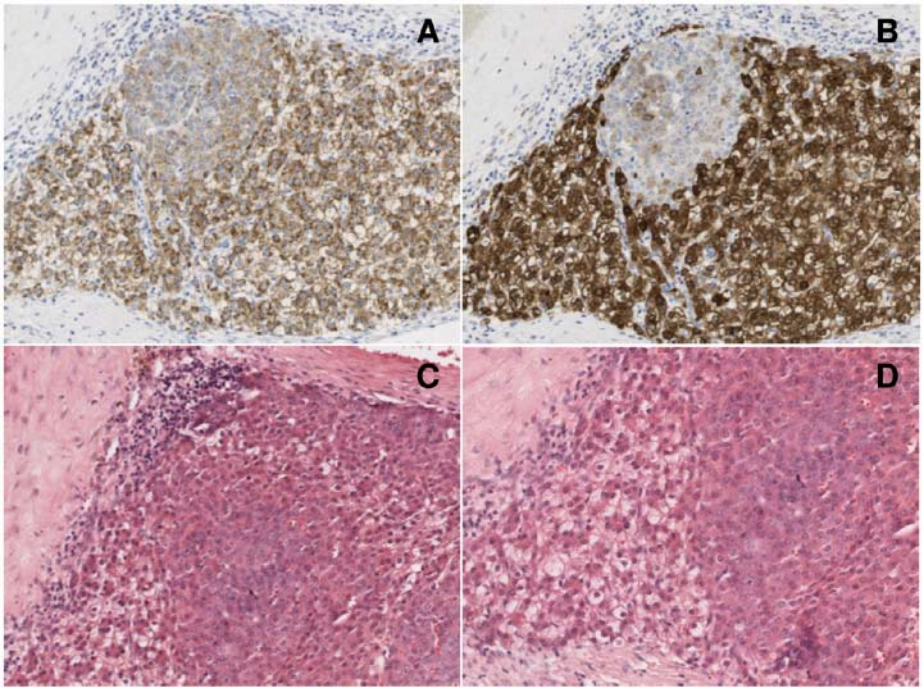

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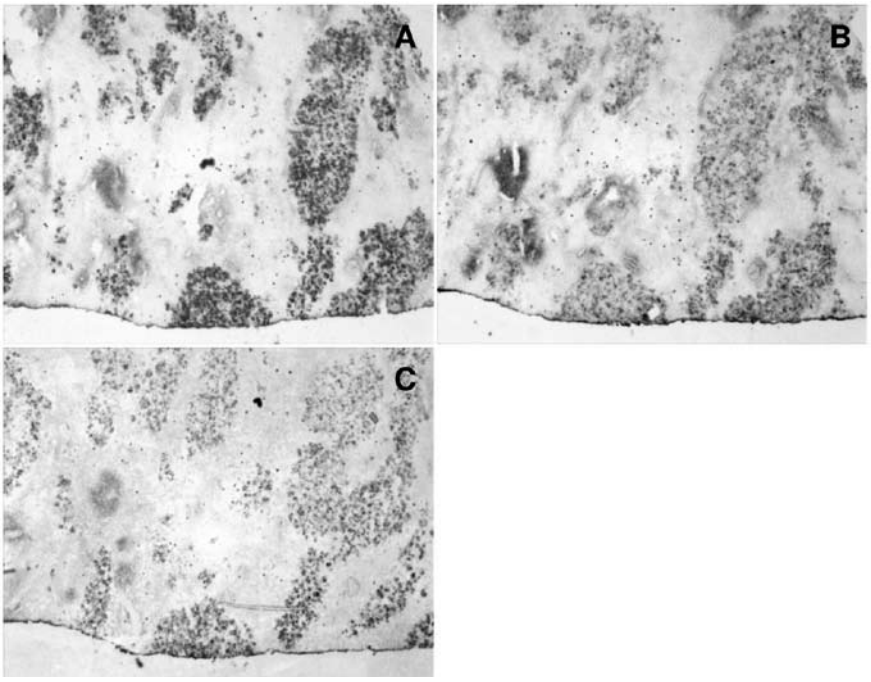

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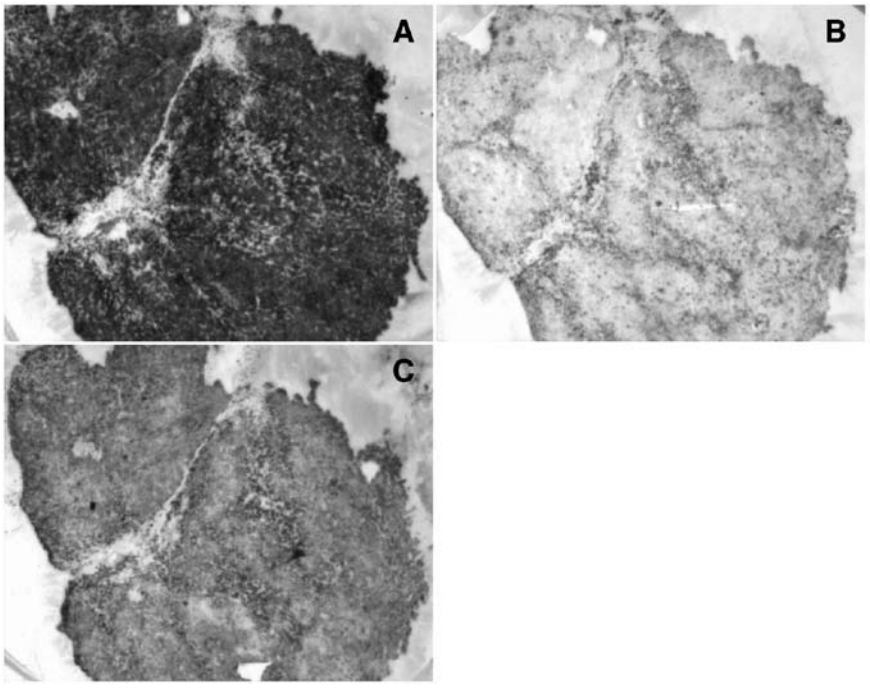

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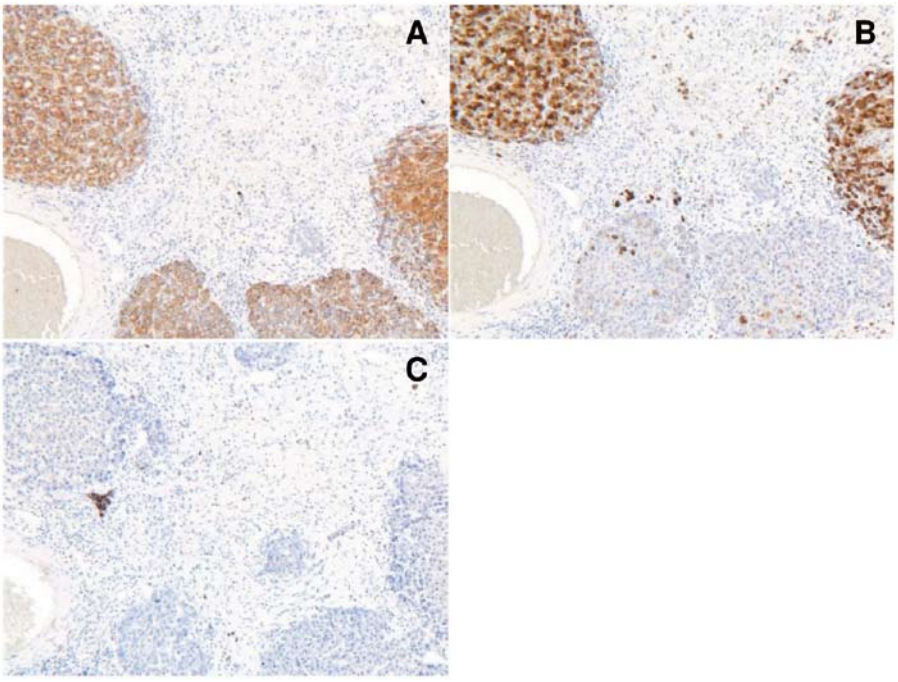

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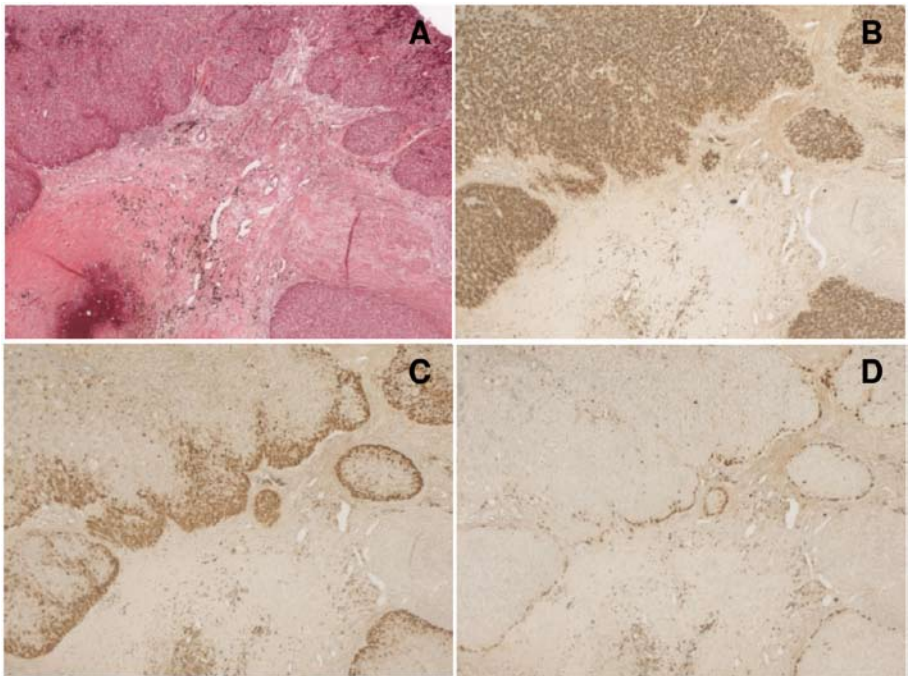

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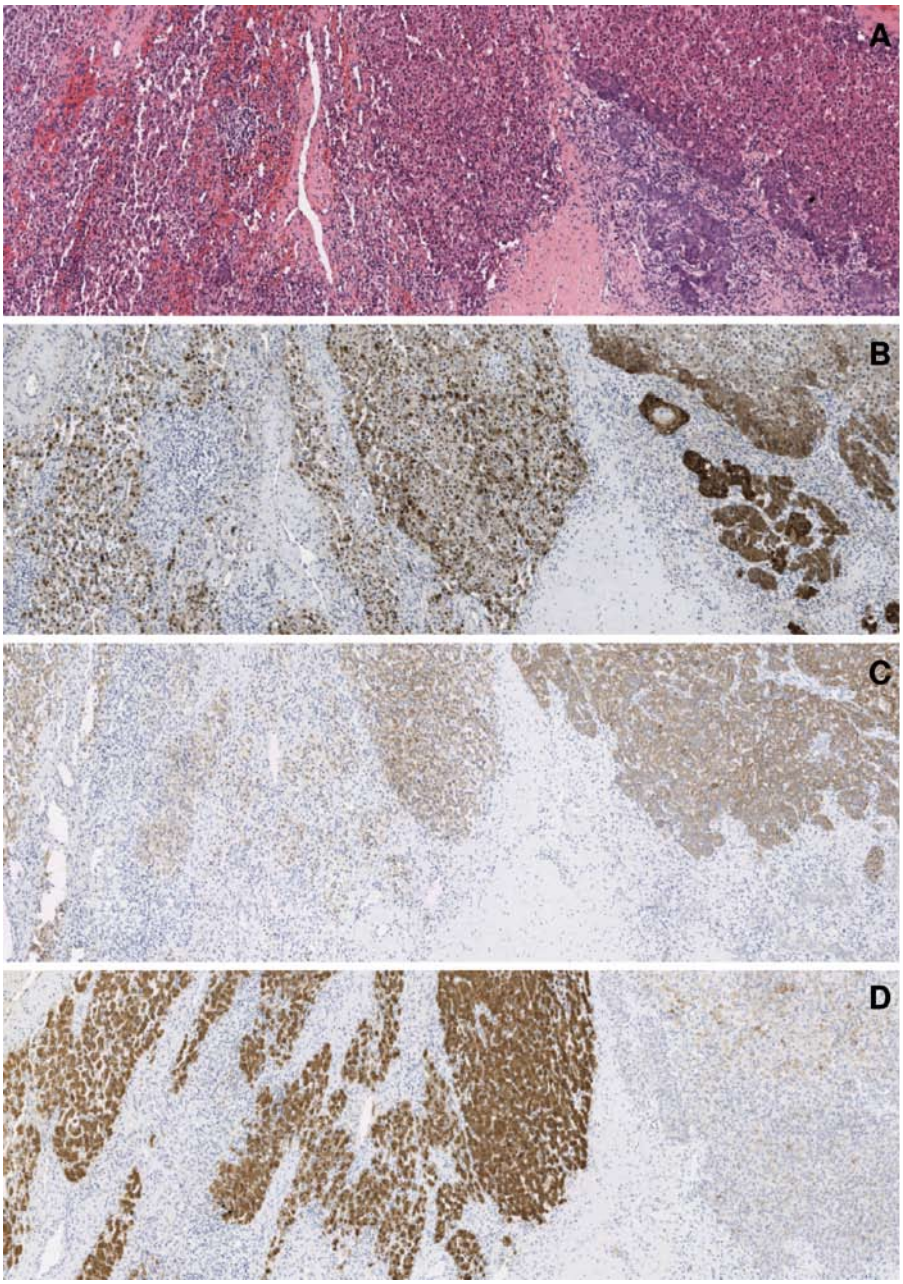

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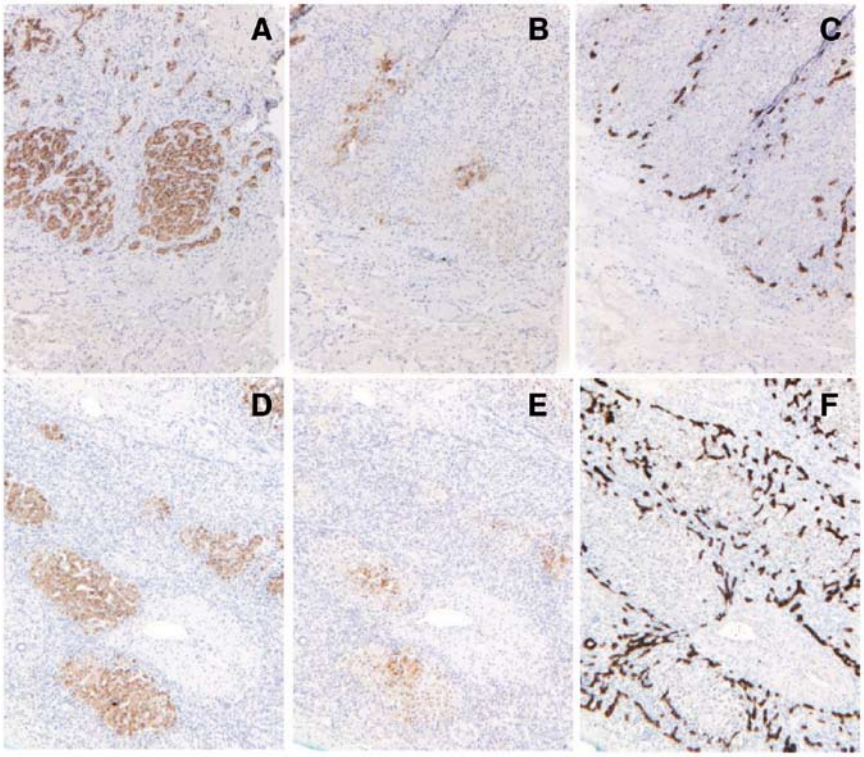

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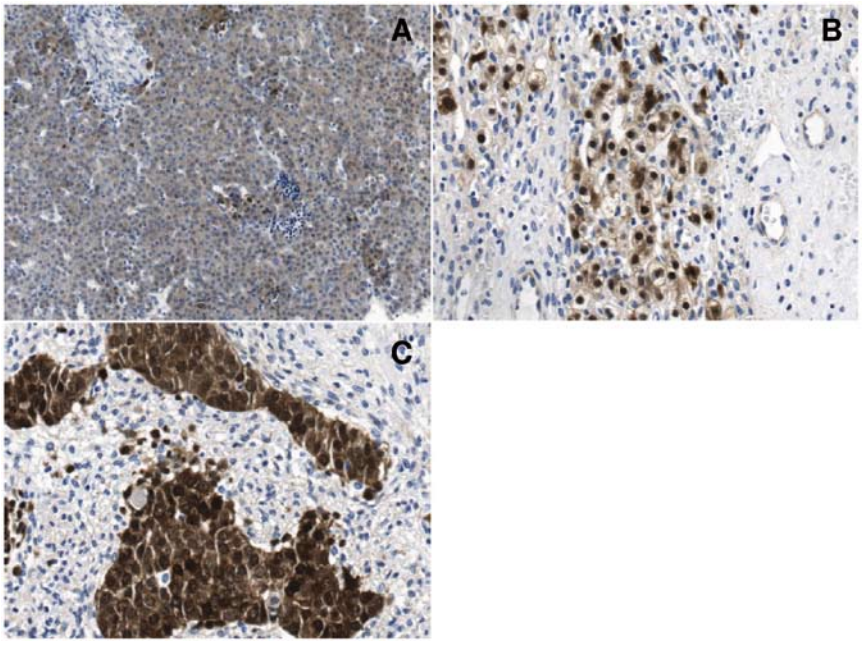

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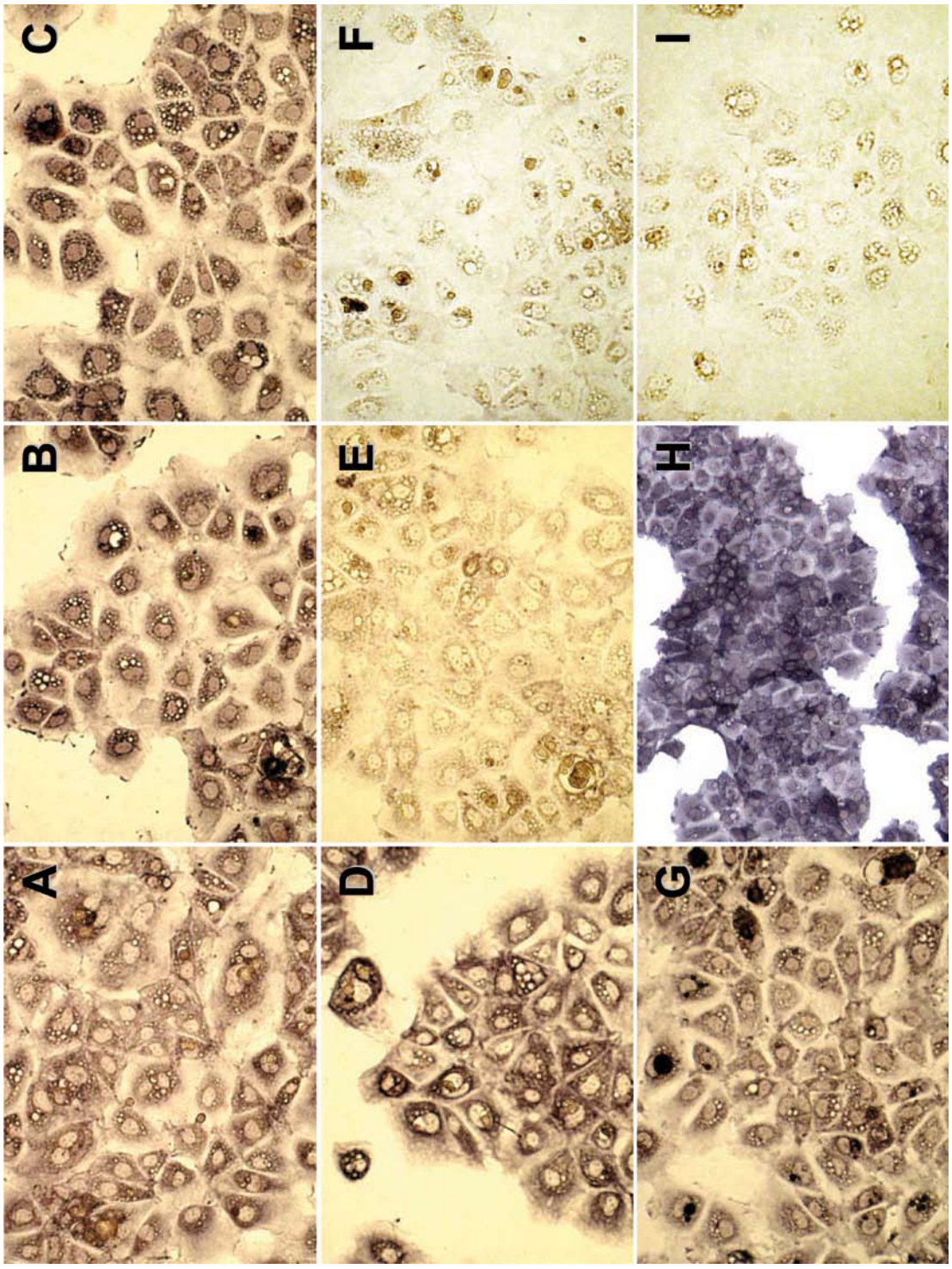
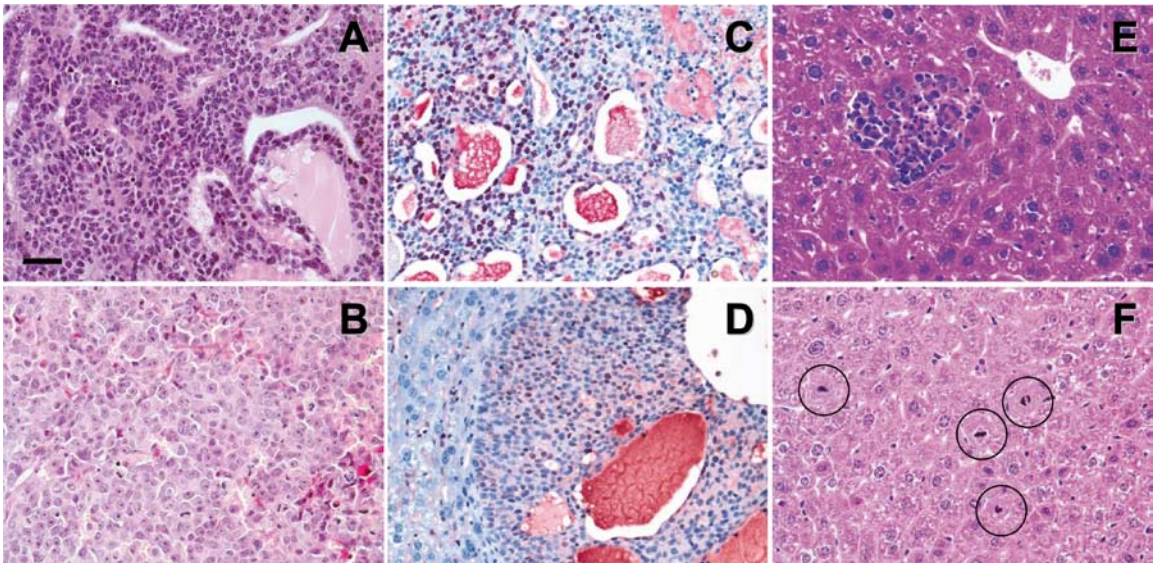
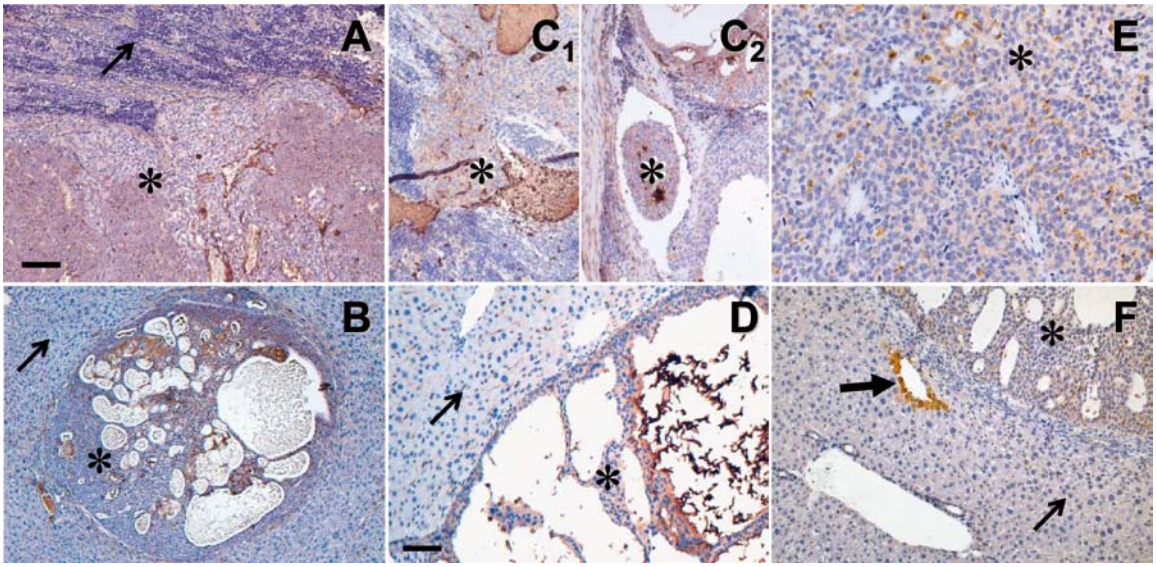


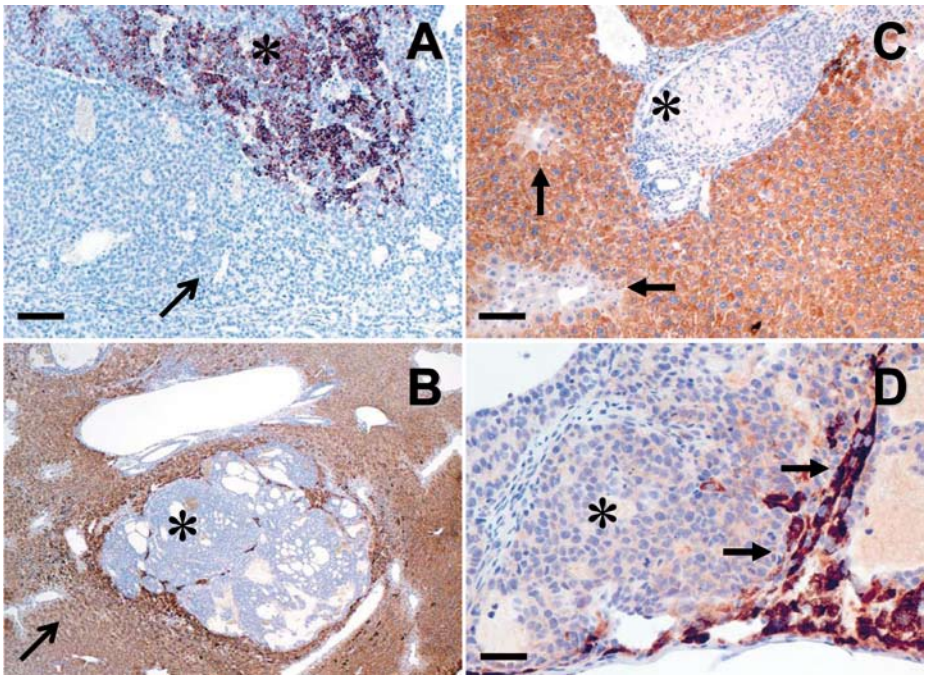
Chapter 6 **Figure 1**



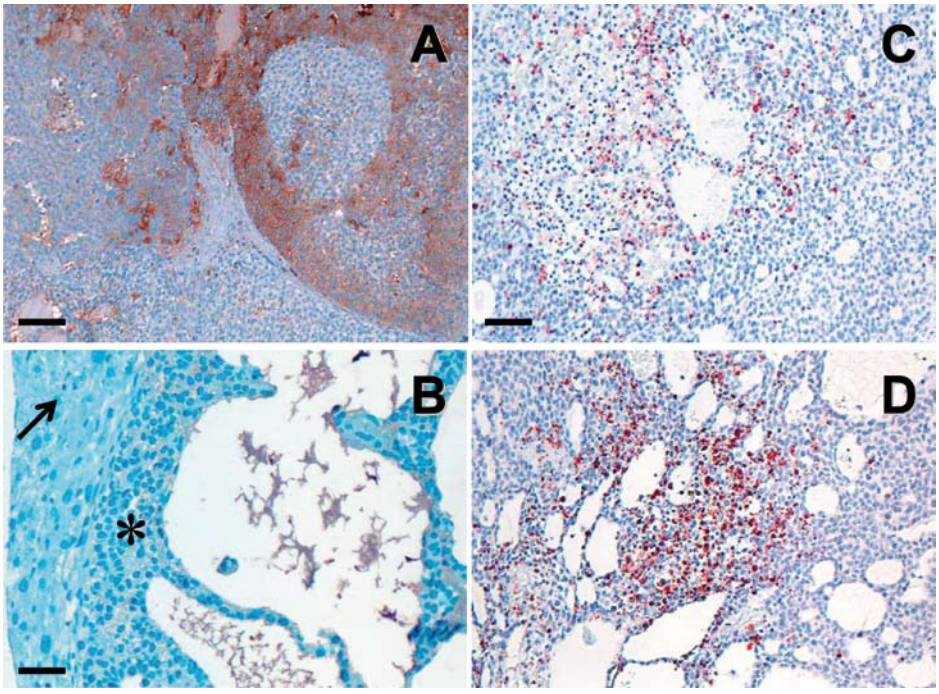
Chapter 6 **Figure 2**



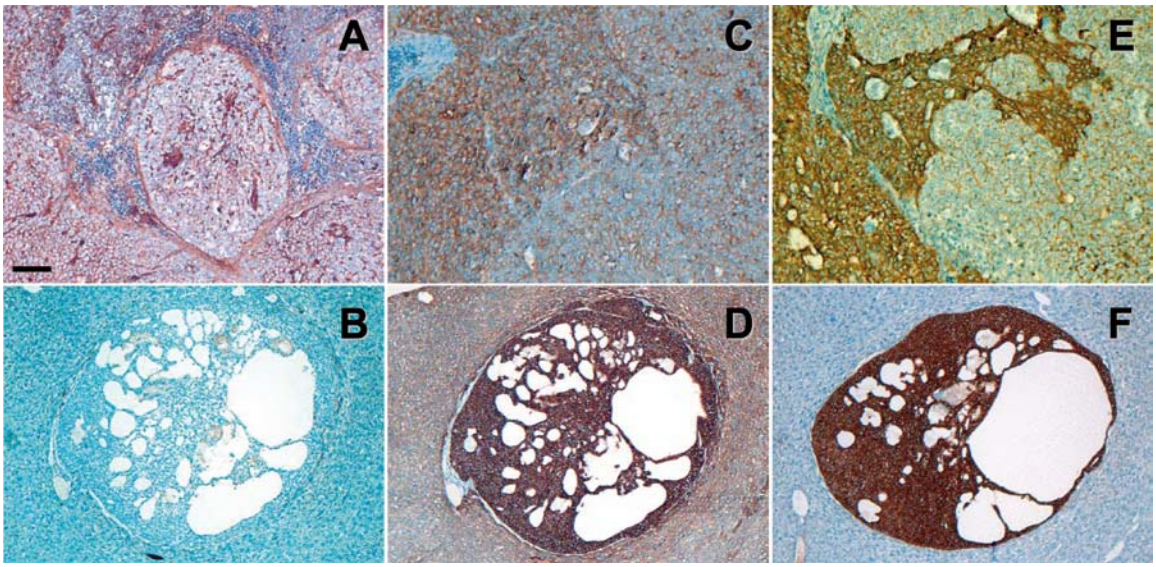
Chapter 6 **Figure 3**



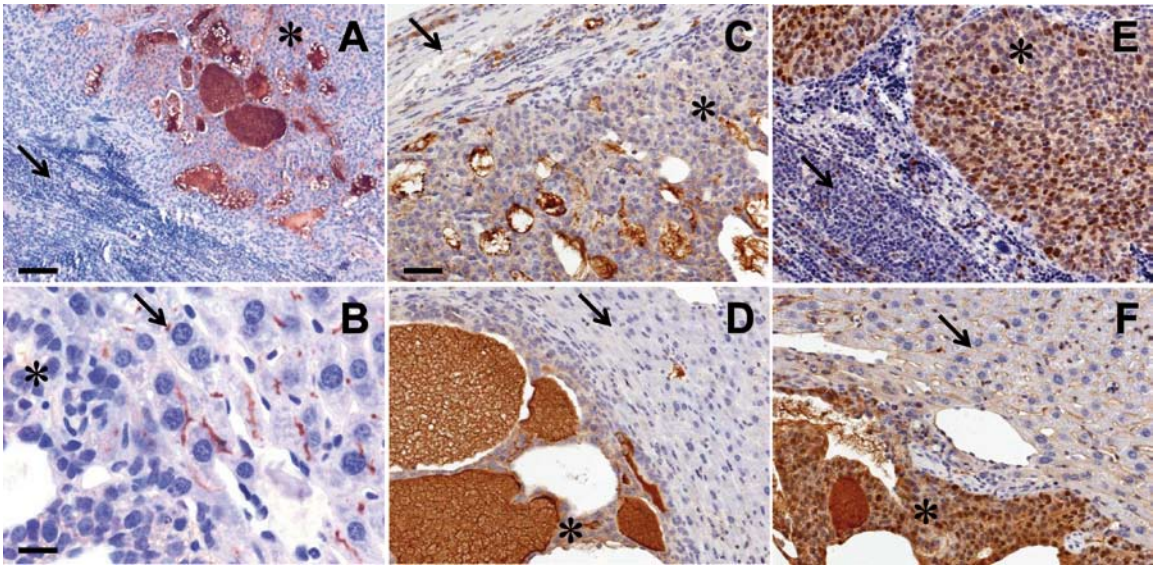
Chapter 6 **Figure 4**



Chapter 6 **Figure 7**



Chapter 6 **Figure 5**



Chapter 6 **Figure 6**

Dankwoord

Dankwoord

Tot slot van dit alles ben ik dank verschuldigd aan de mensen die hebben bijgedragen aan de totstandkoming van dit proefschrift.

Mijn promotores. Professor dr. W.H. Lamers, beste Wout, de allereerste schreden op zoek naar de basaal wetenschappelijke achtergronden van het hepatoblastoom werden in jouw laboratorium gezet. Je wetenschappelijke visie, kritische blik, tomeloze energie en gedrevenheid zijn van dien aard dat ik me geen betere promotor en begeleider had kunnen wensen. Ik heb het als een voorrecht beschouwd om onder jouw bezielende leiding te mogen werken en ben je nog steeds dankbaar voor je bereidheid een klinisch onderzoeker onder je hoede te nemen. Tijdens mijn buitenlandse perioden zorgden jouw e-mail onderschriften 'de kaasboer' en 'Der Wout' er altijd voor dat ik met weemoed terugdacht aan die mooie tijd in 'dat kleine landje' en liet je me menig maal zien waar een klein land groot in kan zijn.

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Mijn co-promotor. Dr. D.C. Aronson, beste Daniël, sinds de allereerste dag dat ik bij jou aan de deur klopte heb je me enthousiast gemaakt voor 'het doen van onderzoek'. Jouw betrokkenheid, doorzettingsvermogen, werklust en wetenschappelijke output dragen er mede toe bij dat het kleine groepje onderzoekers binnen de kinderchirurgie dit alles succesvol kunnen doen. Het vertrouwen dat je me de afgelopen jaren hebt gegeven door mij te betrekken bij de SIOPEL-1 studie groep en jouw rol in de verschillende buitenlandse onderzoeksplekken hebben mij niet alleen wetenschappelijk maar ook als mens gevormd. De gezellige tijden die we samen hebben meegemaakt op congres, tijdens vergaderingen, in de trein of het vliegtuig, maar ook de gastvrijheid bij jou en Liesbeth thuis maakten dit een onvergetelijke tijd.

De promotiecommissie. Prof. Dr. T. Pietsch and Prof. Dr. D. von Schweinitz, es war eine Ehre für mich, in den Labors der Personen zu arbeiten, die für ihre Arbeiten über Hepatoblastome berühmt sind; und ich bin dankbar, dass es Ihnen beiden möglich war, zu meiner Promotionsfeier zu kommen. Dr. J. Bras, dr. T.B.M. Hakvoort, prof. dr. T.M. van Gulik en prof. dr. A. Vos, dank voor uw bereidheid zitting te willen nemen in de promotiecommissie.

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wilde hebben. Ik wist overigens niet dat een geïmporteerd blikje Heineken zo'n enorme vreugdedans teweeg kon brengen...

Kees, de tijd die wij de afgelopen jaren in het ziekenhuis, de auto, de kroeg of op het sportveld hebben doorgebracht zorgden ervoor dat er een vriendschap is ontstaan die verder gaat dat het woord collega. Jouw bereidheid om als paranimf te fungeren, ondanks de emoties die dat misschien teweeg brengt, gaven dit alles nog meer betekenis en maakten het voor mij des te waardevoller. Dank!

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De afdelingen. Allereerst natuurlijk het Kinderchirurgisch Centrum Amsterdam. De gezelligheid, collegialiteit, motivatie en goede sfeer die zowel in het AMC als de VU door jullie wordt uitgedragen en de supermooie stage heeft er mede voor gezorgd dat ik er nooit spijt van heb gehad voor een kinderchirurgisch onderwerp te kiezen. Wat mij betreft wordt de samenwerking nog lang gecontinueerd. De nauwe banden die in de loop der jaren met andere afdelingen binnen het AMC zijn ontstaan hebben deze onderzoeksperiode een extra dimensie gegeven. De afdeling pathologie wil ik bedanken voor hun bijdrage aan de immunologische studies en de afdeling Klinische Epidemiologie en Biostatistiek (KEB) voor de statistische analyses.

The SIOPEL-1 study group. I would like to thank all the members of the Liver Tumor Study Group of the International Society of Pediatric Oncology for giving me the opportunity to be part of and analyze the data of the first prospective trial (SIOPEL-1) that was launched.

De chirurgen en assistenten. Eindelijk is het dan zo ver, de woorden 'het is echt bijna af' zijn omgezet in daden. Alle (oud)assistenten en chirurgen van het Albert Schweitzer ziekenhuis in Dordrecht en het AMC ben ik dank verschuldigd voor de mooie en leerzame tijd die ik tot nu toe heb gehad. Ik ben mij er terdege van bewust dat het volgen van de opleiding tot chirurg in opleidingsregio II iets is om trots op te zijn en dat het garant staat voor een goede opleiding. Zonder jullie steun, collegialiteit en vertrouwen was dit alles onmogelijk.

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Hopelijk ben ik niemand vergeten!

Marco Schnater
Dordrecht 2006

Curriculum vitae

Curriculum vitae

Johannes Marco Schnater werd op 6 januari 1971 geboren te Amsterdam. Na het behalen van het VWO-diploma in 1991, werd de studie geneeskunde een jaar later begonnen aan de Universiteit van Amsterdam. Tijdens zijn studie werd er stage gelopen in Frankrijk en India. Tussen 1994 en 1999 werd als student-assistent bij de afdelingen Huisartsgeneeskunde en Anatomie & Embryologie aan de Universiteit van Amsterdam gewerkt. Tijdens de co-schappen begon hij aan het promotieonderzoek onder begeleiding van dr. D.C. Aronson van het Kinderchirurgisch Centrum Amsterdam - Emma Kinderziekenhuis/AMC en prof. dr. W.H. Lamers, afdeling Anatomie & Embryologie van de Universiteit van Amsterdam. Na het behalen van de artsenbul in 1999, werkte hij als arts-assistent chirurgie/orthopaedie in het BovenIJ ziekenhuis in Amsterdam, waarna het promotieonderzoek werd voortgezet aan de Wilhelm-Friedrich-Universität in Bonn, Duitsland en in het Universitäts-kinderspital beider Basel te Basel, Zwitserland. Op 1 april 2001 begon hij zijn opleiding tot chirurg in het Albert Schweitzer ziekenhuis te Dordrecht (opleiders dr. K.G. Tan & dr. R.J. Oostenbroek) waar hij voorzitter van de Arts-Assistenten Vereniging was. In 2005 vervolgde hij zijn opleiding in het Academisch Medisch Centrum (AMC) te Amsterdam (opleiders prof. dr. D.J. Gouma & prof. dr. J.J.B. van Lanschot) en was hij vertegenwoordiger van de arts-assistenten in opleidingsregio II. Na afronding van zijn opleiding tot algemeen chirurg in 2007 zal hij de vervolgopleiding vaatchirurgie in het AMC beginnen onder leiding van dr. R. Balm en prof. dr. D.A. Legemate.

Publicaties

Publicaties

1. Schnater JM, Sleeboom C, Raaymakers EL, Ekkelkamp S, Aronson DC. [Femoral shaft fracture in children younger than 4 years: shorter hospital stays with the help of at home traction apparatus]. *Ned Tijdschr Geneeskd* 1998; 142(23):1324-7. Comments in: Ned Tijdschr Geneeskd 1998; 142(35):1970; Ned Tijdschr Geneesk 1998; 142(39):2166-7.
2. Schnater JM, Aronson DC, Plaschkes J, et al. Surgical view of the treatment of patients with hepatoblastoma: results from the first prospective trial of the International Society of Pediatric Oncology Liver Tumor Study Group. *Cancer* 2002; 94(4):1111-20.
3. Schnater JM, Köhler SE, Bruder E, et al. Ein Tiermodell für das humane Hepatoblastom. In: Siewert JR, ed. Chirurgisches Forum 2002 für experimentelle und klinische Forschung., vol. 31, ed. 1. Berlin: Springer-Verlag, 2002; 543-7.
4. Schnater JM, Köhler SE, Lamers WH, Von Schweinitz D, Aronson DC. Where do we stand with hepatoblastoma? a review. *Cancer* 2003; 98(4):668-78.
5. van der Vlies CH, Schnater JM, Tutein Nolthenius RP, Coenen JMFH, Tan KG. Een gecompliceerd beloop na een 'eenvoudige' appendicitis bij een kind. *Ned Tijdschr Heelkd* 2003; 12(4):129-30, 144.
6. Schnater JM, Plaisier PW, vd Berg PM, Schütte PR. A remarkable outcome after video-assisted thoracoscopic resection of a giant bulla. *Interact Cardiovasc Thorac Surg* 2003; 2(4):589-91.
7. de Jong VM, Schnater JM. [Diagnostic image (193). A man with a palpable mass in the upper abdomen]. *Ned Tijdschr Geneeskd* 2004; 148(24):1198.
   Comments in: Ned Tijdschr Geneeskd 2004; 148(37):1844.
8. Schnater JM, Eland EM, van der Straaten F, Plaisier PW. Lung cancer presenting as a giant tumor of the thoracic wall. *Eur J Cardio-Thorac Surg* 2004; 25(6):1122-3.
9. Schnater JM, Plaisier PW. Predictors of improvement in health-related quality of life in patients undergoing cholecystectomy. *Br J Surg_on line* 2004; (bjsH/1,2004,0-4-401-3274-0).
10. Wit EMK, Schnater JM. [Diagnostic image (201). A man with a swelling of a finger]. *Ned Tijdschr Geneeskd* 2004; 148(32):1590.
    Comments in: Ned Tijdschr Geneeskd 2004; 148(44):2202-3.
11. Schnater JM, Schouten - van Meeteren AYN, Heins YM, Aronson DC. Hepatoblastoma in a patient with a partial trisomy 9p syndrome. A case report. *Cancer Genet Cytogenet* 2005; 156(1):77-9.
12. Steunebrink M, Schnater JM, Storm RK, et al. Bilateral axillary metastases of occult breast carcinoma: report of a case with a review of the literature. *Breast* 2005; 14(2):165-8.
13. Schnater JM, Aronson DC, Staalman CR, et al. Predictive value of the pretreatment extent of disease system in hepatoblastoma – Results from the International Society of Pediatric Oncology Liver Tumor Study Group SIOPEL-1 study. *J Clin Oncol* 2005; 23(6):1245-52.
14. Schnater JM, van Zalen-Sprock RM, Schaap AHP, Festen S, Aronson DC. Ileal atresia and thrombo-embolic liver calcifications diagnosed after treatment with intra-uterine laser coagulation therapy for twin to twin transfusion syndrome. *J Ped Surg* 2005; 40(5):875-6.
15. Schnater JM, Kuijper CF, Zsiros J, Heij HA, Aronson DC. Preoperative diagnostic biopsy and surgery in paediatric liver tumours – The Amsterdam experience –. *Eur J Surg Oncol* 2005; 31(10):1160-5.
16. Schnater JM, Idu MM, Aronson DC. Extrahepatische vena portae trombose: de mesenterico-portale bypass ('Rex shunt') biedt uitkomst! *Nederl Tijdschr Heelkd* 2006; 15(5): 138-40.
17. Scheerder MJ, Schütte PR, Schnater JM. Claudicatio intermittens bij een 26-jarige wielrenner. *Ned Tijdschr Geneeskd* 2006; 150(27): 1518-22.
18. Schnater JM, Bruder E, Bertschin S, et al. Subcutaneous and intrahepatic growth of human hepatoblastoma in immunodeficient mice. *J Hepatol* 2006; 45(3): 377-86.
19. Schnater JM, Legemate DA. Vascular surgery (boekaankondiging). *Nederl Tijdschr Geneesk* 2006; 150(32): 1801-2.
20 Schnater JM, Legemate DA. Endovascular treatment (boekaankondiging). *Nederl Tijdschr Geneesk* 2006; *in press*.

J'ai voulu voir, j'ai vu...