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ORIGINAL ARTICLE

Diagnostic and Prognostic Aspects of Hepatocellular Carcinoma – A Retrospective Analysis in 145 Patients

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ABSTRACT

BACKGROUND: Prognosis of advanced hepatocellular carcinoma (HCC) is still poor. In this retrospective study prognostic factors for long-term survival and an immunohistochemical panel for discrimination of HCC from other liver malignancies were analyzed.

MATERIALS AND METHODS: In 181 primary liver tumors clinical data, tumor characteristics and the primary mode of treatment were analyzed using univariate and multivariate statistics. In 156 cases (145 HCC, 36 intrahepatic CCC) the immunohistochemical profile of the tumor tissue using molecular markers as HepPar-1, AFP, CD34, CK7, CK20, CA19-9 and CDX2 was established routinely. Significance of marker expression, sensitivity, specify and positive predictive value of the analyzed markers in relation to histological subtype were estimated using SPSS 10.0.

RESULTS: Median overall survival (OS) was 15 ± 19.2 months. Multivariate analysis identified tumour size ($p = 0.001$), grading ($p = 0.002$), proliferative activity (Ki67 level; $p = 0.032$), multifocal tumour ($p = 0.045$), liver function (Child-Pugh score, $p = 0.045$) and performed tumour resection ($p < 0.0001$) as independent prognostic factors for survival. HepPar-1 was the most frequently expressed marker in HCC (positive in 71.8%; $p < 0.0001$) whereas positive AFP staining was less common (positive in 48.7%; $p < 0.0001$). The CD34 protein as a marker for vascular-associated tissue showed a positive reaction in 54.1% of tissues from HCC patients in comparison to 2 patients (6%) with cholangiocarcinoma ($p < 0.0001$).

CONCLUSIONS: Our data identified tumor stage, tumor biology and performed surgical therapy as independent prognostic factors for OS in HCC. Best predictive markers for differentiation between HCC and CCC were HepPar-1, CK7 and CA19-9. Using this panel fast and accurate differentiation by IHC was possible in more than 95% of the patients.

Key words: Hepatocellular carcinoma; Ki67; proliferative activity; AFP; CK7; Immunohisto-chemistry (IHC)

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer death worldwide[1,2]. In the last few decades, the incidence of HCC has increased, possibly due to the growing worldwide prevalence of chronic hepatitis B or C. Other reasons for human hepatocarcinogenesis include alcohol abuse and metabolic disorders (e.g. hemochromatosis) leading to liver cirrhosis. Exposure to carcinogens (e.g. aflatoxin plays a minor role[3]. Especially in developed countries, the emergence of hepatitis C virus (HCV) and chronic liver damage and inflammation due to steatohepatitis are increasingly prominent etiological factors for human hepatocarcinogenesis[4,5]. Likewise, incidence of HCC increased in Germany up to 9.2-10.7/100.000 in men and 1.6-3.6/100.000 in women.

HCC is characterized by poor prognosis leading to the second most frequent cause of cancer-related mortality, and has the shortest survival time of any human cancer. Without therapy, patients with HCC normally die within 12 months, due to rapid progression [6,7]. Currently, surgical resection and liver transplantation are the best available treatment options for HCC[8-10], Tumor resection including partial hepatectomy is widely accepted as the first treatment option for many HCC patients[11]. Liver transplantation is significantly more laborious due to the lack of donor organs with long waiting periods, higher perioperative risk, and long-term immunosuppression. Despite surgical treatment options long-term prognosis of HCC is disappointing due to a high incidence of recurrence (68%-96%[12]) leading to 5 year overall survival rates below 30% in operated patients and in the range of 5-7%[13,14] inpatients without tumor resection. Tumor characteristics that determine the biological aggressiveness and metastatic potential of the disease may be important predictors of survival and hence important elements in the evaluation of HCC [15,16].

Differentiation of HCC from other liver tumors as cholangiocarcinoma (CCC) is in clinical routine a frequent diagnostic dilemma for pathologists. However, accurate diagnosis is crucial, because treatment options differ considerably: In contrast to CCC, HCC is chemo-resistant. Liver transplantation is a potential therapeutic option in patients with HCC and liver cirrhosis but usually not recommended in patients with CCC[17,18]. In addition to hematoxylin and eosin (H&E) staining several immunohistochemical markers for distinction of liver tumors are used in clinical practice. However, utility of each of these markers is limited either by suboptimal sensitivity or difficulty in interpretation of results, especially in poorly differentiated tumors or scirrhous hepatocellular carcinoma[19-21].

In this retrospective single center study we analyzed the impact of histopathological findings and of immunohistochemical markers for diagnosis and prognosis of HCC of different etiologies in a German patient population.

PATIENTS AND METHODS

Patients

Clinical and histopathological data of 145 patients with HCC (72.4% males; mean age 68.5 ± 9.9 years; for details see Table 1) and histopathological data of 36 patients with cholangiocarcinoma (CCC) were analyzed in this retrospective study. Our cohort includes patients who were admitted to the Marienhospital Stuttgart between January 2004 to February 2014.

Histopathological evaluation

Pathological reports included clinical data and information on tumor typing, staging (according to UICC 2002), and grading (WHO graduation G1–3). Hematoxylin-eosin staining was performed to detect features such as bile canalicular structure and Mallory hyaline bodies. Histochemical staining and immunohistochemical staining was performed with antibodies against HepPar-1, AFP, CK7, CK20, CDX2, CA19-9, CD34, CEA on all resected tissue biopsies to confirm the histological diagnosis of HCC or CCC and to exclude other types of malignancy.

A mouse monoclonal anti-human Ki-67 antibody (Anti-Ki-67/MIB1 (Hu); ultraView Universal DAB Detection Kit Roche Diagnostics, Grenzach-Wylen, GERMANY) was used on paraffin embedded sections for immunohistochemical analysis of proliferative capacity. Only distinct nuclear staining of carcinoma cells was used for scoring via the light microscope, determined semiquantitatively as nil (no immunostaining), low (10% or less immunopositivity), mean (> 10-20%) or high (> 20%) immunoreactive cells, respectively. The entire tumor represented in the section was assessed.

Serum analysis of alpha-fetoprotein (AFP) in patients with HCC

In 97 patients with HCC (80.8%) serum levels of AFP were analysed using chemiluminescence technique (CLIA, Immulite AFP, Siemens Healthcare, Germany). A cut off elevation ≥ 200 ng/mL was chosen to be regarded suspicious for HCC since also chronic viral infection or inflammation may lead to elevated AFP serum levels.

Treatment modalities of hepatocellular carcinoma (HCC)

For patients with potentially resectable HCC the Barcelona classification (BCLC) was used for treatment decision. In 32.4% of all cases [n = 47; HCC UICC I–III; sufficient liver function (Child A-B)] a surgical tumor resection (atypical or hemihepatectomy) with lymph node dissection was performed routinely.

In 24.8% of patients (n = 36) with multifocal tumor, insufficient liver function or other contraindications for surgical treatment chemoembolization using doxorubicin (1-8 sessions; mean 3.8) was performed. 13.1% (n = 19) of the patients were treated with sorafenib, and 29.6% (n = 43) with best supportive care (BSC) only.

Statistical analysis

Associations between histopathological findings such as tumor differentiation, proliferative activity (Ki67), tumor stage and survival rates were analysed using t test and 2-sided Fisher´s test. Impact of clinicopathological factors on survival were analysed by univariate and multivariate methods. Median overall survival (OS) was estimated according to the Kaplan–Meier method. Differences in survival were analyzed using a log-rank test. Sensitivity, specify and the predictive value of immunohistochemical markers and clinical data were also analysed using SPSS 10.0 (Inc., Chicago, IL) software. $P \leq 0.05$ was considered statistically significant.

Table 1 Clinical and etiological data of the study population (n = 181).

	Gender (male %)	Mean age (years)	Hepatitis (HBV/HCV) (%)	alcohol (%)	Steatohepatitis (%)	Cirrhosis (%)
Cholangiocarcinoma (n = 36)	50	67.5 ± 11.5	5.4	8.1	8.1	13.5
Hepatocellular carcinoma (n = 145)	72.4	68.5 ± 9.9	37.5	43.7	7	73.3
p-value	0.048	n.s.	< 0.001	< 0.001	n.s.	< 0.001

RESULTS

Association of tumor therapy, HCC stage (TNM classification), and liver function with overall survival (OS)

Median overall survival (OS) of all patients was 15 ± 19.2 months. OS correlated significantly with treatment of HCC. After surgical therapy OS was significantly longer (24.5 ± 28.9 months) than in patients without tumor resection (6.5 ± 9.6 months, $p < 0.0001$). OS in patients with small tumor size (T1) was significantly longer (36 ± 22.3) in comparison to patients with T4 tumors with OS of only 7 ± 6.9 months ($p < 0.0001$). Following the 2-year observation period 61.7% of patients with T1 tumors were still alive whereas all patients with T4 tumors had died (Figure 1). After 5 year follow-up the overall survival rates of patients with T1 tumor dropped down to 34.8%. In patients with advanced liver disease and reduced liver function (Child-Pugh B/C) OS dropped significantly after 2 years as compared to patients with Child-Pugh A liver function (8.5%% *vs* 48.4%; $p < 0.001$).

OS of patients with singular tumor manifestation was significantly longer (14.5 ± 24.5 months) as compared to OS in patients with multifocal tumors (7.5 ± 9.1 months; $p = 0.012$). OS of patients who had metastatic disease at diagnosis was similarly short (6.5 ± 12 months).

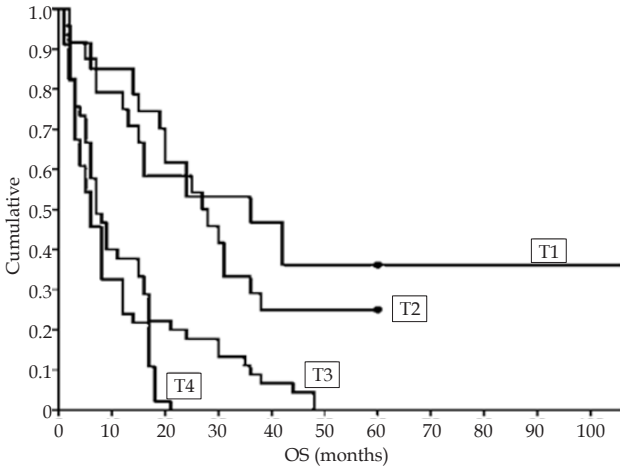

Figure 1 Correlation between tumor stage (T1-4) and OS ($n = 145$).

Association of tumor differentiation with tumor stage (TNM), multifocal tumor and proliferative activity (Ki67)

Tumor differentiation correlated significantly with tumor stage and proliferative activity. In larger tumors (T3, T4), incidence of poorly differentiated tumor cells was 31.1% and 81.2%, whereas in T1 and T2 tumors only 0% or 8.3% dedifferentiated tumor cells were detected ($p = 0.0037$; see Figure 2). Tumor cell differentiation was also significantly associated with multifocal HCC nodes (45.5% in G3 tumors *vs* 17.6% in G1 tumors; $p = 0.0003$). In G1 tumors Ki67 labelling was significantly lower (12.7 ± 17.6%) when compared with G2 (20.6 ± 21.2%; $p = 0.001$) and G3 tumors (42.5 ± 14.1%; $p < 0.0001$; see Figure 3).

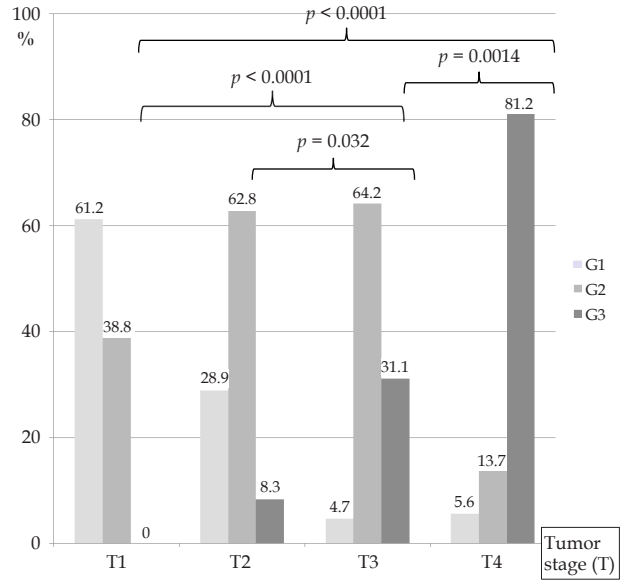

Figure 2 Correlation between tumor cell differentiation (grading; G1-3) and tumor stage (TNM). Poorer differentiation was significantly correlated with more advanced tumor stage.

Multivariate analysis of prognostic factors in HCC

Multivariate analysis identified tumor size (T stage; $p = 0.001$), grading ($p = 0.002$), proliferative activity (Ki67 level; $p = 0.032$), liver function (Child-Pugh score; $p = 0.045$), multifocal tumor stage ($p = 0.045$) and performed tumor resection ($p < 0.0001$) as independent prognostic factors for survival.

Immunophenotypic Profile of Hepatocellular Carcinoma

HepPar-1 positive in 71.8% of HCCs and was the most frequently expressed marker in this disease. In CCC the marker was detectable in 3% only ($p < 0.0001$). AFP staining was positive in 71 HCCs (48.7%; $p < 0.0001$) and also in one patient with CCC. In this patient histological examination showed a combined hepatocellular-cholangiocarcinoma (combined HCC-CCC) with > 50% of malignant cholangiocytes. The CD34 protein as a marker for vascular-associated tissue showed a positive reaction in 65 patients (54.1%) with HCC in comparison to 2 patients (6%) with cholangiocarcinoma ($p < 0.0001$).

31 patients (86.9%) with intrahepatic cholangiocarcinoma expressed CK7 and 18 (50%) expressed CK20. These results were significantly different from classical hepatocellular carcinoma (CK7 and CK20 expression in 24.3% and 0%, respectively; $p < 0.001$ and $p < 0.0001$). Except for one patient with combined HCC-CC (see above) AFP and HepPar-1 were negative in all patients with HCC.

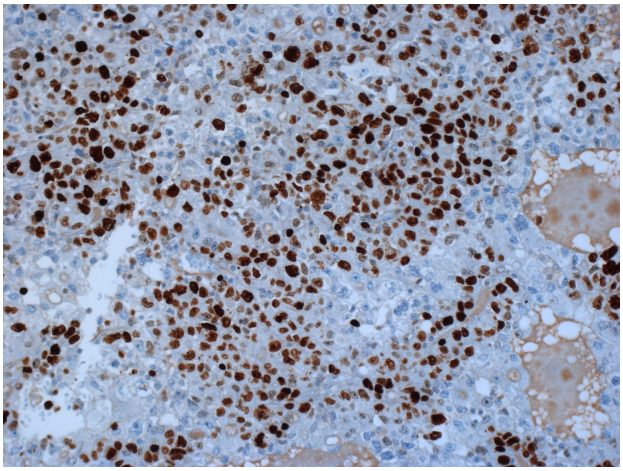

Figure 3 Immunohistochemical staining of a G3 tumor with high labelling of intranuclear Ki67 (~50%; X200)

For more details see Table 2, Figure 4 and Figure 5a, b.

Serum levels of tumor marker AFP and correlation with IHC

In 36% of patients with HCC serum tumor marker AFP was significantly elevated. When compared with the AFP staining in the liver a significant correlation between IHC and serum AFP levels was detected. Increased serum AFP was detectable in only 14.3% of the patients without AFP expression in IHC. Mean AFP serum concentration in these patients was 105 ± 315 ng/mL. In HCC with positive AFP expression in IHC, serum levels were significantly elevated in 63.6% ($p < 0.001$) with a mean serum level of 2129 ± 1661 ng/mL (see Figure 5c).

DISCUSSION

In the present study, overall survival of 145 patients with HCC was analyzed in relation to different risk factors for tumor recurrence and cancer-related death. In addition, an immunohistochemical panel was examined to differentiate HCC from CCC.

Despite new diagnostic approaches and novel therapeutic modalities as tyrosine kinase inhibitors, the prognosis of advanced HCC still remains poor. In our study, overall survival median OS

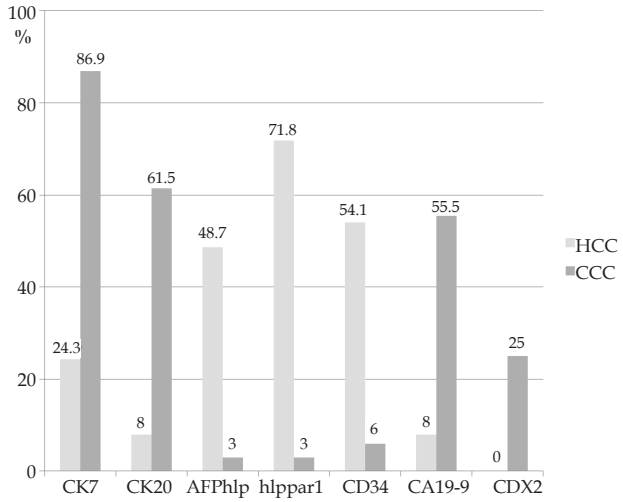

Figure 4 Most frequently expressed markers (%) used for immunohistochemical staining in this study for differentiation of HCC in comparison to CCC.

Table 2 Sensitivity and specify of the most expressed markers for discrimination of HCC and CC using IHC ($n = 181$).

	AFP	HepPar-1	CD34	CK7	CK20	CA19-9
Hepatocellular carcinoma ($n = 145$)*	48.7	71.8	54.1	24.3	0	8
Cholangiocarcinoma ($n = 36$)#	3	3	6	86.9	50	55.5
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Sensitivity	0.48	0.72	0.54	0.86	0.5	0.55
Specify	0.972	0.927	0.944	0.75	1	0.916
pos. predictive value	0.98	0.98	0.97	0.51	1	0.66

Abbreviations: CEA, polyclonal antibody to carcinoembryonic antigen; HepPar-1, Hepatocyte Paraffin 1; CD34, Cluster of differentiation 34; CK7, cytokeratin 7; CK20, cytokeratin 20; CA19-9, carbohydrate antigen 19-9. Numbers reflect percentages. In some cases, available tissue was insufficient to perform all stains. Numbers of patients with analyses performed were *HepPar-1, $n = 125$; CD34 and CK7, $n = 115$; CK20, $n = 90$. # CK20 and CA19-9, $n = 30$; AFP and HepPar-1, $n = 25$.

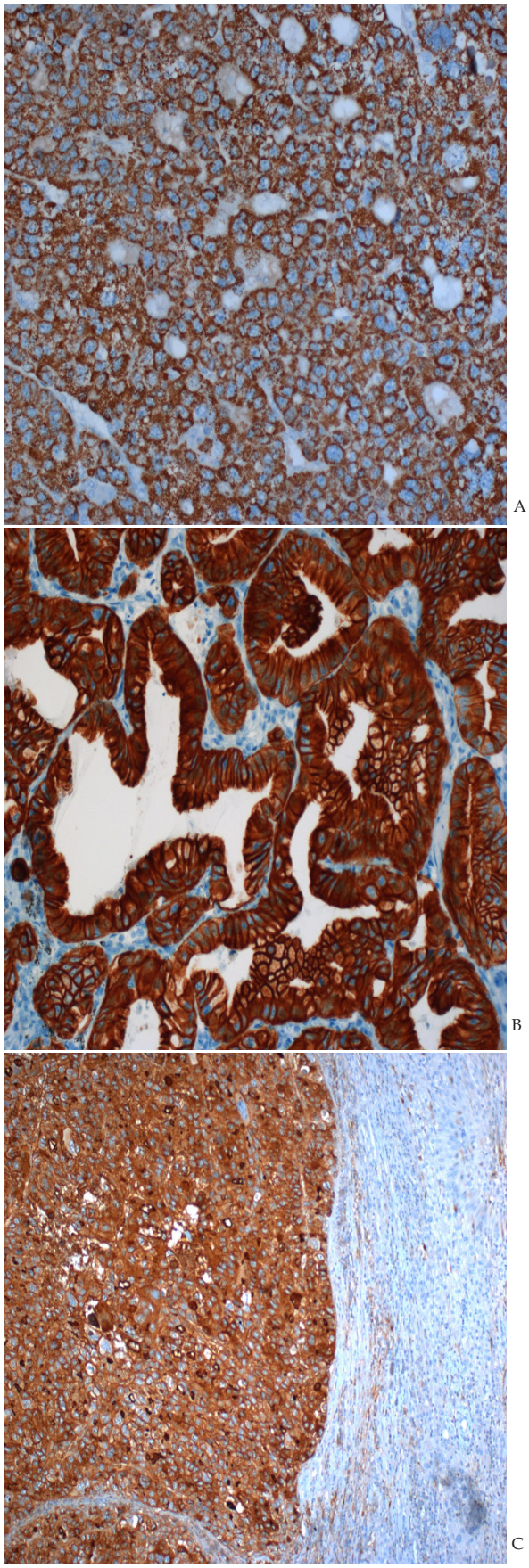

Figure 5 Immunohistochemical staining of the most strongly overexpressed markers for A: HCC, reactivity against HepPar-1(71.8%; x200); B: Cholangiocarcinoma, reactivity against CK7 (86.9%; x200). C: strong reactivity against AFP in a HCC with high AFP serum levels (5245ng/mL).

was 15.0 ± 19.2 months. Whereas in other human cancers prognosis usually depends on tumor stage and aggressiveness survival of most patients with liver cancer is also affected by the underlying chronic liver disease (i.e. cirrhosis and reduced liver function). As shown previously, reduced liver function and end-stage liver cirrhosis (in most cases documented using Child-Pugh or BCLC score) correlate with OS and are a significant predictors for survival of patients with HCC[22,23]. In our study, OS was also significantly lower in patients with reduced liver function (Child B/C) when compared with patients with nonrestrictive liver function (Child-Pugh A), probably due to the fact that only a minority of patients (32.2%) had surgical treatment. It is not surprising that tumor stage at diagnosis is one of the strongest prognostic factors since R0 resection or liver transplantation is the only treatment options offering long-term survival or cure. Possibly due to the underlying liver disease 5-year OS dropped down to 34.8% even in the group of T1 patients with primarily favourable prognosis. As demonstrated earlier[24] multifocal tumor stage was another adverse prognostic indicator of overall survival (7.5 ± 9.1 months; $p = 0.012$), perhaps due to the underlying liver disease, e.g. cirrhosis, favouring multicentre occurrence[25].

Dysregulation of the balance between proliferation and cell death represents a pro-tumorigenic principle in human carcinogenesis resulting in tumor progression and tumor cell seed with occurrence of metastasis. The Ki67 protein is associated with active cell proliferation and expressed in all phases of the cell cycle, especially in G2/M, except for G0. In our study high proliferative activity (measured by Ki67 labelling index) was significantly correlated with poor tumor cell differentiation ($p < 0.0001$). Similar results were seen in previous studies showing higher levels of Ki67 expression in tumor tissue to be associated with higher tumor grade[26] and early disease recurrence[27] probably as a consequence of tumor evolution and higher tumor aggressiveness[28,29]. Histopathologic and biologic factors of tumor such as tumor size ($p = 0.001$) and multifocal tumor stage ($p = 0.045$), surgical treatment ($p < 0.0001$), liver function ($p = 0.045$), cell differentiation ($p = 0.002$) and proliferative activity ($p = 0.032$) were detected as independent prognostic factors using multivariate analysis for OS in patients with HCC.

Diagnosis of Hepatocellular carcinoma (HCC) may be difficult especially in well and poorly differentiated HCC. In well differentiated tumors distinction from normal or regenerative tissue may be very difficult in some cases, whereas some of the unusual morphologic variants, including clear-cell, pleomorphic, and sarcomatoid variants or poorly differentiated tumors, may be mistaken for metastases[20,21].

In our study all tumors could be differentiated by immunohistochemistry, but sometimes only after a second look using the whole immunohistochemical panel and also taking into account clinical reports and serum markers such as AFP or CA19-9. In our cohort typical risk factors for HCC such as viral hepatitis, alcohol abuse, underlying liver cirrhosis and male sex were represented significantly more often in patients with HCC in comparison to the group with cholangiocarcinoma. Thus clinical data and patient history are still important to support clinical diagnosis of primary liver cancer.

Immunohistochemical markers including AFP, HepPar-1 or CD34 are useful to distinguish between HCC and CCC but sensitivity is low (i.e. 0.48 for AFP and 0.71 for HepPar-1). Serum levels of AFP correlated well with the results of IHC but, again, sensitivity was low for detection of HCC. Similar data of a low sensitivity (20-60%) and better specificity (76-96%) of AFP in HCC were published earlier[30,31] leading to the current recommendation by the German Association for the Study of the Liver (GASL)[32] not to use AFP for screening of HCC. HepPar-1 is a very important immunohistochemical marker for HCC and our data are in line with previous studies reporting high expression levels of HepPar-1 in primary liver cancer[19,33]. However, in HCC with scirrhous morphology, absence of HepPar-1 staining and frequent positivity of adenocarcinoma-related markers is characteristic and needs to be differentiated from liver metastasis. IHC for CK7 was positive in more than 24% of our patients with HCC. Interestingly, since it is known that CK7 is expressed in hepatic progenitor cells (HPCs) but normally not in hepatocytes, CK7 positive HCCs potentially derive from HPCs[34]. CK7 is of particular importance to distinguish scirrhous from fibrolamellar HCC, the latter expressing this marker much more often[35,36]. In comparison to CK7, CK20 was expressed only in CCC and thus is helpful to distinguish this tumor from HCC. However, since sensitivity of this marker for CCC is low and since there is stronger expression in colorectal cancer, there may be a pitfall in differentiation of primary liver cancer against secondary tumors of the liver[37]. To solve this problem in clinical routine another immunohistochemical marker with high sensitivity and specify for malignant hepatocytes Glypican-3 (GPC-3) is currently available; however, this was not the case in our retrospective study. GPC-3 is a membrane-anchored heparin sulfate proteoglycan that has been shown to be expressed in approximately 80% of HCC but not in benign hepatic lesions[38,39]. This antigen may also represent a potential therapeutic target[40,41].

There are several limitations to our study. It is a retrospective study and patients with variable treatment modalities, liver function and different etiologies of HCC were included. In most cases (67.8%) histological diagnosis was performed by a needle biopsy only leading to uncertainties in the determination of precise biological characteristics of the tumors, e.g. proliferative activity and tumor cell differentiation neglecting also intraspecific heterogeneity in larger HCCs. Due to the retrospective nature of this study, Child-Pugh score could be estimated in only 78 patients of our cohort.

Our findings are in line with earlier studies[42,43] regarding prognostic factors HCC and emphasize the importance of tumor stage, tumor biology and liver function for long-term survival of these patients. In addition, the present study confirms earlier reports on the complexity of making an accurate diagnosis of HCC and cholangiocarcinoma. For reliable differentiation of primary liver cancer a panel of immunohistochemical markers such as HepPar-1, AFP, CK7 and CA19-9 is essential together with clinical data. Gene expression analysis may open novel perspectives to find better diagnostic and prognostic markers and potential therapeutic targets for primary liver cancer[44,45]. Early data indicate that gene expression profiling may be helpful for subclassification of HCC, cholangiocellular carcinoma and especially combined hepatocellular-cholangiocarcinoma in future clinical routine[46].

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