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„Neue immunhistochemische und molekulare Entwicklungen in der Hepatokarzinogenese “

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1. ***Introduction:***

1.1. Hepatocellular carcinoma (HCC):

1.1.1. ***Epidemiology:***

HCC is the sixth most common type of cancer in terms of incidence, accounting for approximately 630,000 new cases per year in the world; in addition, HCC is the third leading cause of cancer deaths [1].

Worldwide, the biggest risk factors for HCC are the infections caused by hepatitis B virus (HBV) and hepatitis C virus (HCV), which increase the risk of developing liver cancer by about 20-fold [2].

A review of the published literature reveals marked global variation in incidence rates and risk-factor profiles for HCC. Studies of migrant populations have shown that although incidence remains high in the first generation immigrants from high-risk areas, subsequent generations show a decrease in HCC rates. Although the incidence of HCC continues to rise, it is expected that vaccination programs will reduce the rate of HBV-related HCC and that public health measures will do the same to HCV-related HCC in the coming years. In parallel, health education programs may have the potential to curb the rise and impact of diabetes, obesity, and nonalcoholic steatohepatitis (NASH), which are becoming increasingly important HCC risk factors [3].

1.1.1.1. *GLOBAL VARIATIONS IN THE INCIDENCE OF LIVER CANCER AND HCC*

HCC is one area of oncology warranting further investigation into its epidemiology. Although HCC was one of the first cancers to be linked epidemiologically to a definite risk factor (hepatitis B virus in Taiwan) [4], the explanation for what accounts for the incidence of this disease is less clear. Although liver cancer is the sixth most common neoplasm worldwide, its very poor prognosis makes it the third leading cause of cancer-related mortality, responsible for about 600,000 deaths annually [5]. In most countries, HCC accounts for 70-85% of primary liver cancer cases [6], with the burden of disease expected to increase in coming years [7].

Publications arising from analyses of GLOBOCAN database have highlighted striking global variations in the incidence of liver cancer, with the burden of

disease largely concentrated in countries with developing economies [5, 8]. In the most recently published GLOBOCAN global analysis, it was estimated that, in 2002, 82% of liver cancer cases occurred in developing countries, with 55% in China alone [5]. Figure 1 presents GLOBOCAN estimates for age-standardized incidence rates of liver cancer in 2002, grouped by region and gender. Areas where the incidence of liver cancer is moderately high (11-20 cases per 100,000 male inhabitants) or very high (>20 per 100,000) include China, southeastern Asia, and sub-Saharan western and eastern Africa [5, 9]. In most developed areas of the world, including North America and much of Europe, the liver cancer incidence is at low (<5 per 100,000) or intermediate (5-10 per 100,000) levels; however, southern Europe and Japan are notable exceptions, with higher incidence rates (11.6 per 100,000 and 23.1 per 100,000, respectively). Potential reasons for these variations in incidence rates are discussed later. In addition to highlighting geographic trends in incidence, Figure 1 illustrates how rates of liver cancer differ according to gender. Liver cancer is much more common in men than in women; indeed, according to the GLOBOCAN estimates for 2002, the overall male: female incidence ratio was 2.4, and this ratio was even higher in areas of greater HCC risk [5].

HCC is the predominant primary liver cancer in most countries so that the global variations in rates of liver cancer discussed above can be considered an accurate reflection of HCC incidence [6] with some exceptions. For example, in parts of the Philippines and Thailand, biliary tract infection with liver flukes is common, and therefore rates of cholangiocarcinoma are particularly high [5, 6].

Table 1 summarizes the findings of recently published epidemiologic studies that have estimated HCC incidence in the general population of different countries, most commonly via analysis of data held in cancer registries.

Consistent with the findings of the GLOBOCAN analysis of liver cancer incidence, reported rates of HCC are lower in North America and northern Europe than in southern Europe and Japan [10].

1.1.1.2. *HCC RISK-FACTOR EPIDEMIOLOGY*

HCC is a complex disease associated with many risk factors and cofactors [9, 11]. In most patients, HCC is preceded by cirrhosis of the liver [7] and, unsurprisingly, common causes of cirrhosis have been identified as key risk

factors for HCC. Of particular importance is chronic infection with HBV or HCV. Indeed, it has been estimated that HBV is responsible for 50%-80% of HCC cases worldwide, whereas 10%-25% of cases are thought to be a result of HCV infection [12, 13]. To date, eight HBV genotypes (A to H) have been classified [14]. Patients with HBV genotype C have a higher risk for developing HCC, and a genetic mutation has been identified in some of these patients that may contribute to this greater risk [15, 16]. In patients with HBV (genotype D) and HCV coinfected HCC, significantly lower HBV viremia levels were demonstrated than in single-infected HCC individuals [17]. HBV and HCV were found to have an intrahepatic synergistic effect, and the replicative efficiency of HBV was modified by the introduction of genomic instability by HCV [17]. Antiviral therapy resulting in viral suppression is known to significantly decrease the risk for HCC in patients infected with HBV and advanced hepatic fibrosis [18].

Other environmental and genetic risk factors are excessive alcohol consumption, aflatoxin intake, diabetes, obesity, and hereditary hemochromatosis [9, 19].

Data emerging from epidemiologic studies have led to the realization that global variations in HCC incidence largely reflect geographic differences in the prevalence of different disease risk factors [19- 21]. For example, there is marked geographic variability in HBV prevalence, which is reflected across the world: <2% of the population of North America and northern and western Europe are chronic carriers of the HBV surface antigen (HBsAg), compared with >8% of the population of Asia and sub-Saharan Africa [22-24]. The large Taiwan Prospective HBV Study robustly demonstrated that HBV is the primary driver of the high HCC incidence rates in regions of high HBV endemicity [4].

Another potential contributor to the high incidence of HCC in Asia and sub-Saharan Africa is dietary exposure to aflatoxin [25], which is produced by a fungus of the genus Aspergillus and is a common contaminant of foods such as peanuts, grain, legumes, and corn [9]. Aflatoxin appears to act with chronic HBV infection [9] as a cofactor for HCC, further increasing the risk for disease.

HIV infection is also thought to increase rates of HCC in HBV-infected individuals [26], and this may be of particular relevance in sub-Saharan Africa, where about 67% of all individuals living with HIV reside [27].

In countries where HBV infection is not endemic, HCV and alcoholic cirrhosis

are generally considered to be the most important risk factors for HCC [21]. A recent Japanese nested case-control study showed that HCV infection and excessive alcohol consumption contributed to 62% and 17%, respectively, of HCC cases observed in 1970-2002 in 20,000 atomic bomb survivors [28]. Furthermore, in an analysis of SEER registry and Medicare data in the U.S., alcohol-induced liver disease and HCV infection were the most common risk factors, present in 22% and 21%, respectively, of 1,325 patients diagnosed with HCC in 1996-1999 [29]. Other factors, such as diabetes and nonalcoholic fatty liver disease (NAFLD), may be important contributors to the development of HCC in the U.S. [29]. Diabetes was also associated with enhanced HCC risk in the Japanese case-control study described above, as was obesity (body mass index >25 kg/m2) [28]. Both diabetes and obesity are implicated in the development of nonalcoholic steatohepatitis (NASH), the severest form of nonalcoholic fatty liver disease (NAFLD) that is believed to lead to HCC via progression of cirrhosis [9, 30, 31]. The growing burden of diabetes and obesity worldwide may drive future increases in HCC incidence, particularly in developed countries where, to date, the impact of the obesity epidemic has been most marked [32].

1.1.1.3. *PAST AND FUTURE TRENDS IN HCC EPIDEMIOLOGY*

In recent years, a number of epidemiologic studies have highlighted how HCC incidence rates may change over time; for example, analysis of data from the SEER registries demonstrated that HCC incidence rates tripled in the U.S. between 1975 and 2005 [33]. This has been partially attributable to an epidemic of HCV infection in the U.S. during the 1960s [33, 34]. A peak in HCV infection rates was also one of the proposed reasons for a considerable increase in the incidence of HCC observed in southern Europe between 1983 and 1992, along with growing levels of HBV infection and alcohol consumption [35]. Rising rates of obesity and diabetes are thought to have contributed to the increasing incidence of HCC over recent decades in Canada, the U.S., and parts of Europe [36, 37]. The burden of HCC is also growing in Latin America, which was previously known for low rates of liver cancer [5, 38]. In Mexico, for example, general mortality rates for HCC increased from 4.1 per 100,000 in 2000 to 4.7 per 100,000 in 2006, with the impact of HCC on morbidity and

mortality predicted to increase further in the future [38].

However, the incidence of HCC is not increasing everywhere. In the Osaka prefecture of Japan, age-standardized HCC incidence rates in men aged 50-79 years peaked at 41.9 per 100,000 in 1987, remained stable until 1995, then steadily decreased to reach 24.0 per 100,000 in 2003 [39]. This decrease in incidence rates may be explained by the fact that HCV began to penetrate Japan in the 1920s, much earlier than in other countries such as the U.S., and that disease control measures initiated in Japan in the 1950s and 1960s have already led to a reduction in HCV infection rates [39].

Control measures, such as the screening of blood and blood products, and the identification and counseling of infected individuals have the potential to reduce global HCV infection rates [40, 41]. However, the spread of HCV among i.v. drug users is increasing and may partly counteract any anticipated reductions in HCC incidence arising from the decreasing incidental medical transmission of HCV [42].

At present, treatment options for HCV are effective in a minority of patients. An understanding of spontaneous HCV clearance and determination of the optimal time for treatment initiation are just two of the requirements for the development of new therapeutics for HCV [43]. Currently, the recommended therapy for chronic HCV infection is a combination of pegylated interferon-α and the purine analog ribavirin [43, 44]. However, this regimen has limited efficacy and is associated with significant toxicity [43].

HBV vaccination programs were initiated in many East Asian and Asia-Pacific countries during the late 1980s [42, 45, 46] and have begun to reduce mother-to-infant transmission of HBV, the key factor driving chronic HBV infection [46, 47]. Despite these successes, the full impact of such programs on HCC incidence is unlikely to be felt for at least two decades because of the high prevalence of chronic HBV infection in many developing regions and the prolonged lag period between HBV infection and the development of HCC [48]. In addition, the HBV vaccine is ineffective in about 5% of healthy people and that population does not develop immunity to HBV [49, 50].

Although it is difficult to accurately predict future changes in disease epidemiology, some experts have suggested that the overall global incidence of HCC will continue to rise in the next few years until a plateau is reached in

2015-2020 [7]. Subsequent decreases in the rates of HCC have been predicted, resulting, at least in part, from expected improvements in the control of HBV and HCV infection [42]. However, as the contributions of HBV and HCV diminish, risk factors such as diabetes and obesity may become increasingly important drivers of future HCC incidence trends.

1.1.2. ***Etiology and Pathogenesis:***

Factors important in the pathogenesis of HCC are summarized in Table 2 [51-53]. HCC is highly linked to chronic liver disease, in particular chronic hepatitis B and C infection and alcoholic liver disease. Virtually any condition associated with chronic hepatic injury (and especially with cirrhosis) may predispose to HCC. The annual risk of HCCs developing in a cirrhotic liver is estimated at 1% to 6%, with the risk generally highest in the context of viral sources and hereditary hemochromatosis [54]. As already discussed, obesity, aflatoxin contamination of food and co-infection with HIV in patients with chronic viral hepatitis [55, 56] are other risk factors for HCC.

Although the strong etiologic association of HCC with cirrhosis is well known, the pathogenetic sequence leading to malignancy is less well elucidated. Liver cell proliferation is increased during chronic hepatitis but is often decreased in cirrhosis. Activation of stellate cells in cirrhosis leads to increased production of growth factors and other substances that alter hepatocyte proliferation. Limitation of the regenerative reserve of the liver has been attributed to accelerated telomere shortening in hepatocytes in chronic liver disease, leading to telomere dysfunction and susceptibility to chromosomal alterations [57], which would correlate with the observation of allelic gains and losses of numerous chromosomes in HCC [58]. Specific cell cycle checkpoint abnormalities in HCC include frequent mutations in the third nucleotide of codon 249 of TP53 in HCCs linked to exposure to aflatoxin B1 [57], associated with high levels of chromosomal instability. An alternative pathway of carcinogenesis, with extensive Methylation of CpG dinucleotides in the promoters of cancer-related genes, is associated with β-Catenin mutations but not with high levels of chromosomal instability [59], and is frequently seen in non-HBV-related tumors. HBV infection promotes carcinogenesis by at least three mechanisms: integration of viral DNA into the host genome, leading to

chromosomal instability; insertional mutations at specific sites, leading to activation of genes involved in cell proliferation (implicated in 20% to 40% of HCCs arising in HBV); and ability of expression of HBX viral protein to modulate cell proliferation and inactivate p53 activities [60]. HCV does not integrate into the hepatocyte genome and has not been found to be directly oncogenic.

1.1.3. ***Clinical findings:***

Surveillance of patients with chronic liver disease and cirrhosis has led to detection of tumors at asymptomatic stages in many cases, but many still present at advanced stages. Common presenting symptoms are abdominal pain, fullness or a mass, or worsening of symptoms attributed to cirrhosis. HCC may invade hepatic veins and spread to the inferior vena cava or even into the right atrium, producing right-heart failure as the initial manifestation. HCC rarely presents with initial symptoms attributable to metastatic spread. The most common metastatic sites are hilar and other abdominal lymph nodes, bone, and lung, although spread to less common sites such as gastrointestinal tract, skin, heart, kidney, spleen, and pancreas may be seen on occasion. Although most HCC patients die from cancer progression, an appreciable percentage succumbs to complications from cirrhosis [61]. HCC accounts for about 70% of all malignant neoplasms found in the cirrhotic liver in regions of low incidence. Although up to 30% of HCCs in North America may arise in a normal liver, it is much more likely that a malignant tumor in the normal liver represents a metastasis (2% HCC vs. 98% hepatic metastasis) [62]. HCC in the noncirrhotic liver generally appears at a later stage and with a larger mass than does HCC arising in patients with cirrhosis. Screening and early detection programs for HCC rely on a combination of ultrasonography and serum levels of AFP, and have led to the diagnosis of many small (less than 2cm), asymptomatic HCCs. Although it is difficult to demonstrate a decrease in disease-specific or all-cause mortality because of the need for large cohorts and randomization to screening or no screening [56], screening for HCC is considered appropriate because, even though the cure rate for symptomatic cancers is very low (0% to 10% 5-year survival), early-stage tumors amenable to resection or liver transplantation are associated with 5-year survival rates of up to 50% [56]. AFP, a glycoprotein produced primarily by fetal liver, remains the most useful serologic marker for

HCC, although its limitations are well recognized. Sensitivity ranges from 40% to 65% and specificity from 76% to 96% [63]. A cut off of 20 ng/mL is commonly used, and values about 400 ng/mL are considered diagnostic for HCC. Because patients with chronic viral hepatitis may have elevated serum levels in the absence of HCC, AFP appears to be more useful in the patient with nonviral liver disease. Progressive increase in serum AFP is also suggestive of HCC and warrants further investigation. A variant of AFP differing in its sugar chains, AFP-L3, appears to be more specific for HCC than is total AFP. Serum level of serum des-γ-carboxy prothrombin (DCP) has been suggested as a useful marker, with sensitivities ranging from 28% to 89% and specificities from 87% to 96%; DCP does not appear to be nonspecifically elevated in chronic liver disease. Because DCP and AFP levels do not correlate, combination of both markers may improve accuracy in HCC diagnosis [104]: DCP seropositivity using a sensitive assay correlates with large tumor size and inversely with tumor differentiation [64]. Other promising markers being evaluated include glypican-3, insulin-like growth factor-1 (IGF-1), and hepatocyte growth factor (HGF) [63]. Although serum AFP levels may be elevated in nonneoplastic conditions associated with hepatocyte regeneration, such as acute and chronic viral hepatitis and cirrhosis, levels higher than 400 to 500 ng/mL are rarely seen. In chronic viral liver disease, increases in serum AFP are often episodic and correlate with increased transaminases. Other malignant neoplasms often associated with very high levels (more than 1000 ng/mL) of serum AFP include hepatoblastoma, germ cell tumors containing a yolk sac component, and hepatoid adenocarcinomas arising in various sites, such as stomach or ovary.

1.1.4. ***Pathologic features:***

1.1.4.1. SMALL HEPATOCELLULAR CARCINOMA

1.1.4.1.1. *Macroscopic features:*

Small HCC is defined as measuring less than 2 cm in diameter [65]. It may be indistinguishable from a macro-regenerative nodule (MRN) on gross inspection and may be recognized only at the microscopic level, often arising within a dysplastic nodule (DN). A fibrous capsule is commonly present in lesions larger than 1.5 cm. Smaller tumors have a vaguely nodular appearance with indistinct

borders, and are difficult to distinguish from the surrounding cirrhotic liver. The nodules often bulge above the cut surface, are rarely necrotic, and may be variegated, with green areas corresponding to bile staining and yellow areas reflecting fat accumulation in tumor cells. Morphologic characteristics of some small HCCs change when the nodule attains size of 2 to 3 cm, resulting from development of a well-defined capsule, dedifferentiation, and clonal expansion [66].

1.1.4.1.2. *Microscopic features:*

Small HCCs are nearly all well differentiated, consisting of relatively thin, irregular trabeculae (up to three cells thick) of small, crowded hepatocytes with fatty or clear cell change; increased eosinophilia or basophilia may also be seen. Mallory hyaline may be prominent. Reticulin stain showing loss of reticulin fibers may be helpful in establishing the diagnosis. Acinar and pseudoglandular structures may also be seen [66] but are typically smaller than those seen in moderately differentiated HCC. Small, well-differentiated HCC is distinguished from high-grade DNs by a nuclear density greater than twice normal and by mild but definite nuclear atypia (hyperchromasia, irregular nuclear contours). Nucleoli are often inconspicuous. As the nodule of HCC enlarges to greater than 1 cm, there is clonal dedifferentiation, often in a nodule-in-nodule fashion. The moderate or poorly differentiated foci are always found toward the center of the nodule, with a peripheral component of WD-HCC that diminishes with increasing tumor size. Prominent fatty change, present in 36% of cases [66], often declines when the tumor attains a size of 3 cm or more, and may be related to the inadequate development of arterial blood vessels at early stages of tumor growth. Stromal and portal tract invasion may occur, but vascular invasion is rarely identified.

1.1.4.2. ADVANCED HEPATOCELLULAR CARCINOMA

1.1.4.2.1. *Macroscopic features:*

The macroscopic appearance of advanced HCC varies depending on the presence or absence of cirrhosis and the size of the tumor [51, 52]. Tumors arising in a noncirrhotic liver usually grow as a single large mass, occasionally with satellite nodules (massive or expanding type), whereas those associated

with cirrhosis often grow as multiple discrete nodules (nodular type) or numerous minute indistinct nodules (diffuse type) that may be indistinguishable from cirrhosis (cirrhotomimetic). The tumors are occasionally pedunculated. Staging criteria for primary liver carcinoma (HCC and cholangiocarcinoma) depend on the size and number of the tumor nodules and the presence or absence of vascular invasion [67]. The liver is enlarged by one or more tumor nodules that are fleshy, variegated, with green bile-stained, pale yellow and white areas, and areas of hemorrhage and necrosis. Except for the rarely encountered fibrolamellar and scirrhous variants, HCC is soft, with little fibrosis. Invasion of the portal vein branches or hepatic veins is common in larger tumors. Involvement of major bile ducts, with intrabiliary growth, can lead to obstructive jaundice. Multiple tumor nodules may be due to synchronous primaries (multicentric growth) or may represent intrahepatic metastases from tumor spreading through portal vein branches. Although genetic analysis may be needed to confirm multicentricity, features favoring synchronous primary HCCs include multiple HCCs of different histology; presence of peripheral areas of well-differentiated HCC in multiple nodules; and multiple small HCCs or concurrent small HCC with a classic larger HCC [52].

1.1.4.2.2. *Microscopic features:*

The neoplastic cells and micro architecture of HCC resemble normal liver to a greater or lesser extent

depending on the degree of differentiation, which ranges from tumors so well differentiated that the distinction from hepatic adenoma is problematic, to tumors that are highly anaplastic and show little evidence of hepatocellular differentiation. In 1954, Edmondson and Steiner devised a four tiered grading system [68] based on autopsy data; this was subsequently modified in a large series reported from the AFIP [69], and other similar systems have been proposed [51] (Table 3). The World Health Organization (WHO) classification divides tumors into well, moderately, and poorly differentiated, and undifferentiated grades [52]. Most tumors are moderately differentiated (grades 2 to 3), and more than one histologic grade is often present within a given tumor. Although tumor grade has not universally been shown to have a significant impact on outcome, higher nuclear grade has been reported to

predict poorer survival in HCCs resected with curative intent [70], and higher tumor grade corresponds to positivity on positron emission tomography (PET) imaging [71]. Bile located within neoplastic cells or tubular lumina is pathognomonic of HCC, but it is found in less than one third of cases and is not evident in poorly differentiated tumors. The presence of bile canaliculi is also diagnostic. Other than in HCC, bile and bile canaliculi have been identified only in hepatoid adenocarcinomas, most commonly arising in the stomach [72]. As a rule, typical HCCs do not contain abundant stroma, which explains why HCCs tend to be soft, in contrast to many other carcinomas that induce desmoplastic stromal response. Fibrolamellar carcinoma and the scirrhous pattern of HCC are rare variants that are exceptions to the rule that HCC lacks significant fibrosis. Large, blood-filled cystic spaces or vascular lakes within the tumor may mimic peliosis hepatis (pelioid pattern). The WHO [52] recognizes four architectural patterns in addition to the fibrolamellar variant, and three cytologic variants of HCC.

Histologic patterns:

Multiple histologic patterns are often found in the same tumor. Only the fibrolamellar variant appears to have prognostic significance, but familiarity with these architectural patterns may be helpful in arriving at a diagnosis of HCC:

1) Trabecular (sinusoidal, platelike): This commonly found pattern resembles normal hepatic architecture in that the tumor cells grow in cords or plates separated by vascular channels lined by endothelial cells and Kupffer cells, with little or no supporting stroma. The trabeculae vary in thickness, from only a few cells thick (microtrabecular) to broad structures 20 or so cells thick (macrotrabecular). The reticulin framework is generally reduced or absent.

2) Compact (solid): This variant of the trabecular pattern is seen in 5% to 15% of HCCs. Confluent growth or compression of adjacent trabeculae results in a solid growth pattern, with inconspicuous or obliterated sinusoids.

3) Pseudoglandular (acinar): This pattern is usually admixed with the trabecular pattern, and is rarely seen as the dominant pattern in HCC. It is present (at least focally) in many HCCs and may be mistaken for metastatic adenocarcinoma, cholangiocarcinoma, or HCC combined with cholangiocarcinoma. The spaces represent greatly dilated bile canaliculi or degenerated macrotrabeculae, and

are lined by a single layer of tumor cells. The pseudoglands may appear to be freely floating and are not embedded in fibrous stroma, a feature that helps distinguish this pattern from adenocarcinoma.

The pseudoglandular spaces may contain bile plugs and/ or proteinaceous eosinophilic material that is positive for PAS but negative for mucicarmine and Alcian blue, and does not represent mucin secretion.

4) Scirrhous: This variant accounts for less than 1% to 2% of all HCCs.

Cytologic appearance and variants:

The tumor cells of HCC are usually polygonal, but may be cuboidal or even columnar. They have a moderate amount of finely granular eosinophilic cytoplasm that may be more basophilic than normal liver and usually have distinct cell borders. Bile canaliculi are readily identified in most well- to moderately differentiated tumors but may be inconspicuous by light microscopy in high-grade HCCs. Compared to normal hepatocytes; the tumor cells display a higher nuclear-to-cytoplasmic ratio. The nucleus is usually round to oval, with coarse chromatin, a single prominent nucleolus, and a thickened or irregular nuclear membrane. Intranuclear cytoplasmic invaginations are a common, albeit nonspecific, finding in HCC. A variety of cytoplasmic inclusions may be identified in HCC cells (Table 4 and Figure 2). Fat droplets can be seen in up to two thirds of tumors [51]. Diffuse accumulation of fat or glycogen results in a clear appearance to the cytoplasm, a variant termed clear cell carcinoma, which must be distinguished from metastatic renal cell carcinoma and other clear cell neoplasms. Mallory hyaline, representing clumps of intermediate filaments, is found in approximately 20% of cases [51] and may be very prominent. The accumulation of Mallory hyaline does not appear to be related to the underlying liver disease, although tumor Mallory bodies are indistinguishable from those seen in steatohepatitis and in chronic cholestasis. Globular proteinaceous eosinophilic inclusions are also seen in 20% of cases and are usually PAS-positive, diastase-resistant, representing accumulation of Alpha 1-antitrypsin (A1AT). More lightly staining inclusions (8% of cases) that resemble ground-glass cells of hepatitis B have been termed pale bodies and represent accumulations of fibrinogen. Copper-binding protein (Shikata orcein stain) and copper (rhodamine stain) have been detected in up to 28% of HCCs and appear to be related to the presence of bile within the tumor [73]. Hemosiderin is rarely

found in HCC, even in the context of hereditary hemochromatosis. Calcification is also quite rare in untreated cases. The following categories are recognized cytologic variants of HCC [74]:

•Pleomorphic (giant-cell):

Tumor cells show marked variation in size, shape, and staining characteristics, and bizarre tumor giant cells are common in this rare variant. The tumor cells show marked loss of cell cohesion, and a distinct trabecular pattern is difficult to identify in this high-grade variant. Extensive sectioning may be required to find evidence of typical HCC. Up to 30% of lower-grade HCCs also contain multinucleated tumor giant cells without anaplastic features; these tumors should be graded according to the overall nuclear features [51].

•Clear-cell:

As the name implies, this variant is characterized by tumor cells with prominent clear cytoplasm resulting from accumulation of cytoplasmic glycogen and/ or fat that is lost in the embedding process. Although HCCs predominantly composed of clear cells are seen in only about 16% of cases, foci of clear-cell change are common in otherwise typical HCCs. Differentiating this type from metastatic renal, adrenal, or ovarian carcinoma may be problematic on purely histologic grounds, and the tumor may require extensive sampling to demonstrate foci of typical HCC. IHC studies demonstrating bile canaliculi (polyclonal CEA or p-CEA) and hepatocellular differentiation such as HepPar-1 may be required to establish the diagnosis of HCC. Elevated serum AFP level, presence of chronic liver disease, and the absence of an extrahepatic mass favor a hepatic primary tumor.

•Sarcomatoid (spindle cell, pseudosarcomatous):

Up to 4% of HCCs exhibit a prominent sarcomatoid or spindle cell component [75] characterized by spindle-shaped cells that suggest a diagnosis of fibrosarcoma or malignant fibrous histiocytoma. Sometimes these tumors have been reported as carcinosarcoma or malignant mixed tumors, particularly when foci of more differentiated sarcomas (osteosarcoma, chondrosarcoma, leiomyosarcoma, rhabdomyosarcoma) are noted [51]. In some cases, a transition between the spindle cell component and typical HCC is demonstrated, whereas in others the components are separate [76]; extensive sampling may be needed to demonstrate areas of typical HCC. Pleomorphic and osteoclast-

like giant cells may be seen, resulting in overlap with the giant-cell variant. CK immunoreactivity, most commonly CAM5.2, is detected in the spindle cells in about 60% of cases [76], supporting an epithelial origin, but the spindle cells also express mesenchymal markers such as vimentin, smooth muscle actin, and desmin. S100 can be detected in a small number of cases. The serum AFP level is elevated in roughly half of reported patients, similar to that seen for typical HCC [76]. Survival after hepatic resection appears to be shortened for this rare high-grade variant [76]. Spindle cell change may be more common in tumors subjected to chemoembolization or preoperative chemotherapy [74].

1.1.4.3. *Immunohistochemistry and special stains:*

Although many cases of HCC are readily recognizable because of the characteristic trabecular architecture with little intervening stroma and demonstration of bile production by neoplastic cells, IHC studies and a small panel of special stains may be invaluable in diagnostically challenging cases. Relatively few non-IHC stains are useful in evaluation of tumors suspected to be HCCs. Reticulin stain is often helpful in distinguishing WD-HCC from nonneoplastic or benign nodules because the reticulin framework is typically reduced and incomplete in HCC; the stain also serves to highlight the trabecular structure, and delineates thickened liver cell plates. Because HCCs do not produce mucin, demonstration of intracellular mucin by mucicarmine stain or Alcian blue is useful in distinguishing HCC from cholangiocarcinoma or metastatic adenocarcinoma. A useful panel for initial workup of HCC includes:
(a) polyclonal or cross-reactive CEA antiserum or antibodies directed against CD10 to highlight inconspicuous bile canaliculi;
(b) antibodies against HepPar-1 and AFP to show hepatocellular differentiation;
(c) monoclonal CEA or MOC-31 to highlight metastatic adenocarcinoma; and
(d) synaptophysin and chromogranin to rule out a low grade metastatic neuroendocrine neoplasm. Polyclonal anti-CEA antiserum or certain monoclonal CEA (m-CEA) antibodies that cross-react with canalicular biliary glycoprotein 1 highlight bile canaliculi (canalicular pattern) in 30% to 100% of HCCs in sections and 47% to 83% in cell blocks or cytologic smears [77], depending on the degree of differentiation of the tumor. A similar pattern may be seen with antibodies directed against CD10; although CD10 expression may

be somewhat less sensitive than p-CEA, interpretation is often easier because of less background cytoplasmic staining in the tumor cells. Demonstration of this canalicular pattern of expression of CEA or CD10 staining remains one of the most useful IHC markers in the differential diagnosis of HCC, although utility is less in higher-grade tumors, which tend to show only focal positivity, if any. About 50% of poorly differentiated tumors lack immunoreactivity. A false positive HCC interpretation of a canalicular pattern may result from entrapment of nonneoplastic hepatocytes within the tumor, misinterpretation of an incomplete membrane pattern as canalicular in location, or misinterpretation of periluminal immunoreactivity for p-CEA in adenocarcinomas as staining of dilated canaliculi. HCCs, unlike adenocarcinomas, rarely show cytoplasmic immunoreactivity with m-CEA antisera, and inclusion of such antibodies in a panel may be diagnostically useful.

HepPar-1 is a relatively hepatocyte-specific monoclonal antibody that reacts with a hepatocyte epitope that is resistant to formalin fixation and tissue processing and is present in both adult and fetal liver. The expression pattern is granular and cytoplasmic and may be patchy within tumors. HepPar-1 is useful in distinguishing HCC from cholangiocarcinoma and metastatic adenocarcinoma; it does not distinguish between HCC and benign Hepatocellular proliferations. Sensitivity for HCC in recent series ranges from 73% [78] to 93% [79], with negative cases more likely to show a scirrhous architectural pattern or poor differentiation. Most nonhepatocytic neoplasms are negative for HepPar-1, although up to 6% of nonhepatic tumors in one series [80] were positive. Adenocarcinomas of the gastrointestinal tract, lung, and neuroendocrine carcinomas may express HepPar-1 [80, 81], and, as with many antibodies, HepPar-1 is probably best used as part of a panel. Scattered strongly positive cells in an otherwise negative tumor should be interpreted with caution, as they may represent entrapped hepatocytes. Normal hepatocytes express CK8 and CK18, which theoretically should be useful in differentiating HCC from cholangiocarcinoma (which tends to express CK7 and CK19 and variably express CK20) and metastatic adenocarcinomas. However, CK expression profiles are variable in HCC as well as in other malignancies, limiting enthusiasm for this approach as an initial diagnostic strategy. A panel of CK7, CK20, and CK19 has been suggested as a practical approach [77]; negative

results for all three markers favors HCC, whereas CK19 and CK7 positivity favors cholangiocarcinoma. Results for metastatic adenocarcinomas are variable depending on the primary site.

Other antibodies that may be useful include MOC-31, a cell surface glycoprotein that is expressed in cholangiocarcinomas and metastatic adenocarcinomas but less often in HCC [77], and glypican-3, a heparin sulfate proteoglycan normally expressed in fetal liver. Glypican-3 is reportedly expressed in 84% of HCCs and rarely in metastatic adenocarcinoma and cholangiocarcinoma [82]. Cytoplasmic transcription termination factor-1 (TTF-1) has also been suggested as a relatively specific marker for HCC [83]. Claudin-4, which is expressed by cholangiocarcinomas but not HCC, is also a promising marker that may prove useful [84].

AFP IHC is highly specific but relatively insensitive, with positivity in only 17% to 68% of cases [77]. However, this marker may be useful in poorly differentiated tumors, which may not show a canalicular expression pattern of p-CEA or CD10 or expression of HepPar-1.

CD34 has been used to differentiate between hepatic adenoma and other benign hepatic nodules and HCC; HCC is more likely to show diffuse sinusoidal positivity indicative of capillarization of the sinusoids. Expression in benign hepatic nodules is more limited. However, results must be interpreted with caution because there may be considerable overlap in degree of expression and pattern.

A variety of other antibodies of limited clinical utility have been applied to HCC. These include A1AT and alpha 1-antichymotrypsin (A1ACT), which demonstrate a granular cytoplasmic nonspecific expression pattern; most HCCs express A1AT, but up to 70% of metastatic adenocarcinomas are positive as well [77]. AE1/AE3, CAM5.2, B72.3, inhibin, and factor XIIIa are also poorly discriminatory in distinguishing HCC from metastatic adenocarcinoma and cholangiocarcinoma [81]. In situ hybridization for albumin mRNA appears to be highly sensitive for hepatocellular differentiation, but has not yet gained widespread use in the diagnostic setting. Up to 96% of HCCs are positive [77], as are Hepatocellular adenomas and nonneoplastic liver. IHC for albumin is not useful because its abundance in the serum results in high background.

1.1.4.4. *Fine needle aspiration:*

Aspirates of HCC are generally highly cellular due to the soft texture of the tumor and the lack of reticulin scaffolding, often resulting in a finely granular smear in well-differentiated tumors [85]. Trabeculae or clusters of tumor cells are lined by a variable number of elongated endothelial cells. The tumor cells are polygonal with central hyperchromatic nuclei and variably prominent nucleoli. The nuclear-to-cytoplasmic ratio is typically increased but varies with the degree of differentiation, and isolated stripped tumor cell nuclei are common. Intranuclear cytoplasmic invaginations and various cytoplasmic deposits such as bile, proteinaceous globules, and Mallory hyaline can be identified in cytologic preparations. Clear-cell HCC may be confused with signet cell adenocarcinoma or pleomorphic liposarcoma. Correlation of cytologic findings with the microarchitecture demonstrated on smear preparations [86] and in cell block material is critical to establishing the correct diagnosis in such cases showing variant cytologic features. False-negative diagnoses of HCC are related either to very well-differentiated tumors that are difficult to distinguish from nonneoplastic lesions or from Hepatocellular adenoma, or to poorly differentiated tumors that are difficult to distinguish as hepatocellular in origin. The presence of monotonous nuclear atypia and nuclear crowding and the absence of a reticulin framework in cell blocks may be helpful in the differential diagnosis of relatively well-differentiated HCC versus benign hepatic conditions [87]. Conversely, false-positive diagnoses are usually the result of the presence of reactive or dysplastic hepatocytes in a cirrhotic liver. Reactive hepatocytes generally demonstrate a continuum of morphologic changes, and this variability is helpful in distinguishing reactive from malignant hepatocytes.

1.1.4.5. *Precancerous lesions:*

Dysplastic nodules arising in the cirrhotic liver are considered to be direct precursors of many HCCs. Although the role of large-cell change as a premalignant lesion is disputed [88, 89], small-cell change is considered an important morphologic indication of transformation [59], but is relatively uncommon and rarely seen in needle biopsies.

1.1.5. ***Molecular Biology:***

To date, molecular techniques and DNA analyses have not proved helpful enough to warrant routine use in the diagnostic setting. Overexpression of mutated p53 can be detected by IHC in up to 37% of HCCs [77], but its diagnostic utility is limited by poor specificity, both for distinguishing HCCs from other malignancies and from benign reactive conditions. Comparative genomic hybridization and fluorescence in situ hybridization have shown promise in distinguishing HCC from hepatocellular adenoma, based on the observations that HCCs typically demonstrate a wide variety of chromosomal abnormalities, whereas hepatocellular adenomas demonstrate a more limited number of aberrations affecting different chromosomes [77, 90]. However, these techniques are not currently considered standard of practice for diagnosis.

A new massive anchored parallel sequencing (MAPS)-based study (using next-generation sequencing to isolate and sequence HBV integrants) [91] showed integration of the viral DNA into host chromosomes in most of the HBV-related HCCs. The authors identified 296 HBV integration events corresponding to 286 unique integration sites (UISs) with precise HBV-Human DNA junctions.

HBV integration favored chromosome 17 and preferentially integrated into human transcript units. The targeted genes include 7 genes (PTPRJ, CNTN6, IL12B, MYOM1, FNDC3B, LRFN2, FN1) containing IPR003961 (Fibronectin, type III domain), 7 genes (NRG3, MASP2, NELL1, LRP1B, ADAM21, NRXN1, FN1) containing IPR013032 (EGF-like region, conserved site), and three genes (PDE7A, PDE4B, PDE11A) containing IPR002073 (39, 59-cyclic-nucleotide phosphodiesterase). Enriched pathways include hsa04512 (ECM-receptor interaction), hsa04510 (Focal adhesion), and hsa04012 (ErbB signaling pathway) [91].

A clonal expansion model in HCC development was suggested relying on the fact that fewer integration events were found in cancers compared to cancer-adjacent tissues.

A newly published study [92] using whole genome sequencing of 88 HCCs found β-Catenin to be the most frequently mutated oncogene (15.9%) and TP53 the most frequently mutated tumor suppressor (35.2%).

The Wnt/β-Catenin and JAK/STAT pathways, altered in 62.5% and 45.5% of cases respectively, are likely to act as two major oncogenic drivers in HCC.

This study also identifies several prevalent and potentially actionable mutations including activating mutations of Janus Kinase 1 (*JAK1*) in 9.1% of patients and provides a path towards therapeutic intervention of the disease.
Accordingly, single nucleotide variations (SNV), copy number variations (CNV) and HBV integration data for significantly altered genes and pathways in this study were mapped to classify the HCC into three subclasses, revealing distinct genetic profiles for each subclass. S1 and S2 express high level of genes involved in cell cycle control and cell proliferation. Most S1 and S2 tumors are poorly or moderately differentiated with high rate of recurrence. A subset of S1 and S2 tumors harbours HBV integration into the *MLL4* gene locus. S1 tumors also express high level of genes in immune response and angiogenesis. S2 has the highest frequency of *TP53* mutation and the highest serum AFP level. S3 tumors are well or moderately differentiated with a gene expression profile reflecting normal liver function. In addition, S3 has relatively high frequency of *CTNNB1* and *JAK1* mutations, and HBV integration into the *TERT* gene locus. Integrative analysis of gene expression profiles, genetic alterations and clinical characteristics appears to at least partially explain the observed difference in progression-free survival of these subclasses.

MicroRNAs (miRNAs) are a group of tiny RNAs with a fundamental role in the regulation of gene expression. Aberrant expression of several miRNAs was found to be involved in human hepatocarcinogenesis and correlates with bio-pathological and clinical features of HCC. It looks like, aberrantly expressed miRNAs could be linked to cancer-associated pathways, i.e. up-regulation of mir-221 and mir-21 that could promote cell cycle progression, reduce cell death and favour angiogenesis and invasion. These findings suggest that miRNAs could become novel molecular targets for HCC treatment [93].

1.1.6. ***Differential Diagnosis:***

The primary challenges in differential diagnosis of HCC are distinguishing low-grade HCC from benign hepatocellular proliferations, and in distinguishing HCC from cholangiocarcinoma and metastatic adenocarcinoma. Recognition that HCC is relatively uncommon in the normal liver and occurs much more frequently in the setting of chronic liver disease or cirrhosis can aid in making

the distinction, but the possibility of metastasis when evaluating a malignant liver tumor should always be considered. Differentiation of HCC from metastatic neuroendocrine tumors (NETs) poses special problems because of the trabecular growth pattern of both. NETs can have a conspicuous trabecular or acinar arrangement and focal oncocytic or clear-cell change. The nuclei of NETs typically display a finely stippled chromatic pattern and lack conspicuous nuclei. The background liver is normal or may show nonspecific features indicative of mass effect. Although some metastatic neuroendocrine tumors are associated with prominent fibrous stroma, in others the stroma is delicate and inconspicuous, consisting of a rich capillary network surrounding groups of tumor cells. Calcification may also be noted, a feature distinctly unusual in HCC. Although nearly all hepatic NETs represent metastases (usually from the pancreas or small bowel), rare cases of apparent primary tumors have been described [94]. Diffuse, strong immunoreactivity for neuroendocrine markers such as synaptophysin, CD56, and chromogranin helps to differentiate an NET from HCC, but focal immunoreactivity with these antibodies has sometimes been described in HCC [95]. Markers that detect hepatocellular differentiation, such as p-CEA and HepPar-1, should be included in the panel of antibodies chosen. Ultrastructural confirmation of neurosecretery granules may be helpful in individual cases, but is rarely needed.

1.1.7. ***Treatment, Outcome and Prognostic factors:***

Patients who present with symptoms attributed to HCC usually survive only a few months after diagnosis; median survival in many series is roughly 7 months. Longer survivals have been reported in patients enrolled in a screening program who are able to undergo surgical resection or liver transplantation. Five year survival after resection is often reported as 20% to 30%, but with careful selection of patients (noncirrhotic patients or those with compensated cirrhosis, tumors less than 5cm), 5-year survival rates can exceed 40% [96]. The prognosis of patients with HCC is determined not only by HCC stage but also by the functional status of the underlying liver, and it appears that many patients with HCC may die as a result of progressive chronic liver disease not attributable to tumor growth [61]. Palliation with percutaneous ethanol injection, cryoablation, and transcatheter arterial chemoembolization has been employed

with some success in patients with inoperable cases. The most common causes of death are cachexia, gastrointestinal bleeding, and hepatic failure. Spontaneous tumor rupture accounts for about 10% of deaths. Tumor size, number, and location (one or both lobes) of tumor nodules, the presence of small- or large-vessel invasion, and the presence or absence of cirrhosis are the most important prognostic variables for HCC that the pathologist can identify in resection specimens [67]. The first three are reflected in the tumor-node-metastasis (TNM) staging system (Table 5). In particular, microvascular invasion and nuclear grade may have prognostic significance for HCC reselected with curative intent [70, 95]. The distance of the tumor from the resection margin (less than 1cm, more than 1cm or at inked margin) should also be noted in the surgical pathology report. Liver transplantation is generally limited to patients with solitary HCC 5cm or less, or to up to three nodules each smaller than 3cm. Five-year disease-free survival rates for HCC are roughly 46% in the setting of liver transplantation [97].

1.2. Cholangiocarcinoma (CCC):

1.2.1. ***Epidemiology:***

Cholangiocarcinoma is relatively rare, but high incidence rates have been reported in Eastern Asia, especially in Thailand. The etiology of this cancer of the bile ducts appears to be mostly due to specific infectious agents. In 2009, infections with the liver flukes, Clonorchis sinensis or Opistorchis viverrini, were both classified as carcinogenic to humans by the International Agency for Research on Cancer for cholangiocarcinoma. In addition, a possible association between chronic infection with hepatitis B and C viruses and cholangiocarcinoma was also noted. Countries where human liver fluke infection is endemic include China, Korea, Vietnam, Laos, and Cambodia. The implementation of a more intensive preventive and therapeutic program for liver fluke infection may reduce incidence rates of cholangiocarcinoma in endemic areas [98].

1.2.1.1. *OVERVIEW*

Cholangiocarcinomas are relatively rare, but high incidence rates have been

reported in Eastern Asia, especially in Thailand [99]. CCCs are highly fatal tumours, as they are clinically silent in the majority of cases [100-105].

CCC occurs with a highly varying frequency in different areas of the world. CCC is the second most common primary liver cancer and accounted for an estimated 15% of primary liver cancer worldwide [98]; however, it varies widely by region from 5% in Japan [106] and 20% in Pusan (Busan), Korea [107] to 90% in Khon Kaen in Thailand [99]. Recently, a rising tendency of intrahepatic CCC incidence was reported in Western countries [108-111]. The reasons for this increase are not clear, but some of these increases were attributed to the switch between coding systems going from International Classification of Disease-Oncology-2 (ICD-O-2) to ICD-O-3.

The etiology of CCC in Asian countries appears to be mostly linked to infections, especially infections with the liver flukes Clonorchis sinensis (C. sinensis) and Opisthorchis viverrini (O. viverrini) [112-115]. These liver flukes, two close members of the family Opistorchiidae [116], are food borne trematodes that chronically infect the bile ducts. The disease has been present for more than 2300 years in China as some archaeologists found a large number of C. sinensis eggs in the content of the bowel from an ancient corpse buried at the middle stage of the Warring States Period (475-221 BC)] [117]. Liver flukes induce chronic inflammation leading to oxidative DNA-damage of the infected biliary epithelium and malignant transformation [116]. Chronic infection with either of these two liver flukes is considered to be of major socioeconomic importance to humans and animals in Asian countries such as China, Korea, Vietnam, Laos, and Thailand [116]. Persons with C. sinensis infection were infrequently reported from Singapore and Malaysia, and many of them might have been infected in other countries when traveling or through eating imported fish. However, liver fluke infection occurs in all parts of the world where there are Asian immigrants from endemic areas [118].

The infections with C. sinensis or O. viverrini are now both classified in Group 1 by the International Agency for Research on Cancer (IARC) based on ‘sufficient evidence in humans’ for CCC.

In addition, a possible association between chronic infection with HBV and HCV known to cause hepatocellular carcinoma – and cholangiocarcinoma was also reported by IARC as there is only limited human evidence [119].

1.2.1.2. *RISK FACTORS FOR LIVER FLUKE INFECTION*

The main risk factors for liver fluke infection are:

1) Consumption of raw or undercooked freshwater fish.

2) Poor sanitation: 'lavatories' are built adjacent to fish ponds, resulting in human excrement containing C. sinensis eggs ending up in the pond water in China [116]. In Laos, 95.5% of houses in some rural villages do not have a latrine and the people there use animal and/ or human feces as fertilizer.

3) Fresh water aquaculture is developing rapidly, but food quality controls are not in place [120].

4) Accidental ingestion of C. sinensis metacercariae via hands or utensils: contamination occurs as a consequence of not thoroughly washing after catching and handling freshwater fish in endemic areas [116].

1.2.1.3. *TRANSMISSION*

The definitive host is infected by the liver fluke through ingestion of raw or undercooked (i.e. dried, pickled, or salted) infected fish which contain metacercariae – the infective stage of the liver fluke.

Three types of uncooked fish preparations are noted: (1) koi pla, eaten soon after preparation; (2) moderately fermented pla som, stored from a few days to weeks; and (3) pla ra extensively fermented, highly salted fish, stored for at least 2-3 months. At present, koi pla is probably the most infective dish, followed by fish preserved for <7 days, then pla ra and jaewbhong, in which viable metacercariae are rare [121].

1.2.1.4. *OTHER RISK FACTORS OF CCC*

Both intrahepatic CCC and extrahepatic CCC are well-known complications of primary scleosing cholangitis (PSC) in Western countries [122]. The other known risk factors for CCC include cirrhosis, chronic nonalcoholic liver disease, obesity, and hepatolithiasis. Other rarer conditions associated with the development of CCC are bile duct adenoma, multiple biliary papillomatosis, choledochal cysts, congenital fibropolycystic liver, Caroli's disease (cystic dilatation of intrahepatic bile ducts), and exposure to the radiopaque medium thorium dioxide (Thorotrast) [100-102, 104, 109]. Approximately 90% of patients

diagnosed with CCC do not have a recognized risk factor in Western countries [123].

Among these risk factors, hepatolithiasis is a very uncommon disease in the West; in contrast, intra- and extrahepatic bile duct stones are much more common in Eastern Asia [124]. Similarly, in the cholangiocarcinogenesis of other risk factors, bacterial infection and bile stasis, which are demonstrable in virtually all patients, underlie cholangiocarcinoma development [124-126]. This carcinogenic process is not limited to the intrahepatic lesions.

1.2.2. ***Etiology and Pathogenesis:***

The etiology of CCC is generally not known. Most arise in noncirrhotic livers, although a study in Japan revealed that about 5% of all CCCs occurred in the setting of nonbiliary cirrhosis [127]. Chronic inflammation of the bile ducts and conditions causing biliary stasis are believed to play a role in the development of carcinoma. One of the most recognized associations is -as already discussed- with parasitic infections (C. sinensis and O. viverrini). There is some speculation that the additional presence of dietary carcinogens, such as nitrosamines and aflatoxins, may increase the risk of CCC in the setting of parasitic infection [128-130].

Another recognized association with CCC, comprising less than 5% of cases, includes fibrocystic diseases such as congenital hepatic fibrosis, Caroli disease, and autosomal dominant polycystic liver disease (ADPLD). Rare reports of CCC in association with metabolic diseases, specifically hemochromatosis and A1AT deficiency, are present in the literature [131-133]. Not surprisingly, cases of papillary serous carcinoma (PSC) and primary biliary cirrhosis (PBC) have led to the development of CCC, although perhaps fewer than would be expected [134, 135], and bile duct dysplasia is generally rare in PSC. Cholelithiasis has long been recognized to increase the risk of CCC [135, 136]. The association of hepatitis C virus infection with CCC is increasingly recognized [99], and, finally, toxin exposure such as Thorotrast, alcohol, oral contraceptives, and occupational exposure (metal and asbestos industries) have also been reported in association with CCC [51, 134, 135]. Investigators have elucidated a mechanism of carcinogenesis in the setting of hepatolithiasis, following a multistep progression through hyperplasia, dysplasia, in situ carcinoma, and

invasive adenocarcinoma [137]. A number of genetic alterations have been shown to play a role in the carcinogenesis sequence, including loss of p53, increase in antiapoptotic proteins, and increased angiogenic factors [137]. In pursuing the theory of chronic inflammatory insult leading to cancer, other researchers have found that exposure to hydrophobic bile acids (glycoursodeoxycholic acid) can lead to oncogenic mutations in the biliary epithelium that may progress to malignancy [138].

1.2.3. ***Clinical findings:***

Approximately 85% of patients present with symptoms, including abdominal pain, jaundice, ascites, weight loss, fatigue, anorexia, and nausea/ vomiting [139]. A right upper quadrant abdominal mass can be palpated on physical examination in between 5% and 30% of patients [51, 134, 139]. The most common laboratory abnormalities identified with CCC are elevated serum alkaline phosphatase (74%), elevated total bilirubin (70%), and elevated aspartate amino transferase (85%) [51]. Elevations of serum CEA and CA19-9 in combination are helpful in the clinical differential diagnosis of CCC from HCC. In contrast to HCC, serum AFP is generally normal or only slightly elevated. On radiologic examination, a single, large, homogeneous mass with irregular margins is identified on CT scan. Intratumoral calcification may be seen and delayed tumoral contrast enhancement is highly suggestive of CCC [51, 140-142].

1.2.4. ***Pathologic features:***

1.2.4.1. *Macroscopic features:*

On gross examination, CCC is large, very firm, tan-white, and nodular with infiltrating tan-pink areas. The central tumor is often more white and firm than the periphery, due to the presence of a prominent desmoplastic stroma. The tumor is reportedly more frequent in the right lobe, but may spread throughout the liver. Satellite nodules are frequent (one third of cases). Nodules located under the capsule often show central umbilication. Calcification is common, giving a gritty quality to the tumor; however, necrosis, hemorrhage, and cystic degeneration are seen much less commonly than in HCC. The background liver is not cirrhotic [51].

1.2.4.2. *Microscopic features:*

Microscopically, CCC resembles many adenocarcinomas of other primary sites, and therefore must be distinguished from metastatic tumors. CCC most commonly manifests as a well-to moderately differentiated adenocarcinoma forming tubules or acini with a prominent intervening desmoplastic stroma. The cells are columnar or cuboidal with moderate, finely granular, clear to pale eosinophilic cytoplasm and rare mucin production. The nuclei are generally round to oval, smaller than HCC, and lack the distinct nucleoli of HCC. Moderate pleomorphism may be seen with irregularity of the nuclear membranes; multinucleation is rare. The tumor may occasionally show a nesting or trabecular architecture, with focal cribriform patterns. The background tumor is notably less vascular than HCC. The tumor grows through the sinusoids and can spread through the liver via the portal veins. Perineural invasion is frequent and is seen more easily in the hilum of the liver. The presence of an in situ preneoplastic lesion is debatable; however, intestinal metaplasia, hyperplasia, atypical hyperplasia, and epithelial dysplasia have been recognized. In situ (intraductal) adenocarcinoma has been reported, and is predominantly papillary in architecture. These tumors are similar to villous adenomas elsewhere in the gastrointestinal tract. The term intraductal adenocarcinoma or intraductal cholangiocarcinoma has been used for tumors containing high grade dysplasia. A component of invasive cancer must, of course, be ruled out in these cases. Histologic variants of CCC have been reported, including mucinous, adenosquamous (carries a much poorer prognosis), mucopidermoid carcinoma, clear cell carcinoma, and spindle cell carcinoma. All of these variants are very rare [51, 143].

IHC for cytokeratins, including CK7 and CK19, are strongly positive in CCC. CK20 is positive in a minority of cases, and in combination with CK7 may be helpful in the differential diagnosis of CCC from metastatic adenocarcinoma. CEA, CA19-9, EMA, BER-EP4, and blood group antigens are positive in CCC. HepPar-1 is generally negative in CCC. Neuroendocrine markers may rarely be expressed [51, 143].

1.2.5. ***Molecular Biology:***

Cholangiocarcinogenesis is a multi-step process in which the high amount of cytokines and factors secreted during chronic inflammatory processes triggers and maintains this incidence.

Molecules participating in chronic inflammation promote neoplastic process by damaging protooncogenes, DNA mismatch repair genes/proteins, and tumor suppressor genes involved in cell growth, apoptosis, invasiveness, and neoangiogenesis. The final result is the uncontrolled cell proliferation and invasion.

Mutations involving oncogenes and tumor suppressor genes have been reported in ICC, including p53, p62 c-myc, p21 c-ras, and p10 c-erbB-2.

More recently, Kang et al described p53 mutations in mass-forming CC and K-ras mutations in the periductal extension type [144].

Fava and Lorenzini [145] revealed in a recent study that K-*ras*, p53, p14ARF, p16INK4a, and beta-catenin genes can be mutated during the development of ICC, initially or through aberrant expression of AID (Activation-induced cytidine deaminase) in biliary cells [146].

AID is a member of the DNA/RNA-editing enzyme family, whose production showed to be significantly increased in human biopsies of PSC and ICC-affected patients compared with normal liver parenchyma.

1.2.6. ***Differential Diagnosis:***

The key challenge in diagnosis is differentiating CCC from metastatic adenocarcinoma. Unfortunately, there are no histologic markers that reliably distinguish primary from metastatic adenocarcinoma. IHC is helpful to a certain degree, although the search for the ultimate marker for CCC is still in the works. A panel of CK7, CK20, TTF-1, gross cystic disease fluid protein (GCDFP), and prostate specific antigen (PSA) may help to differentiate tumors such as colorectal, breast, lung, and prostate. HCC may show a pseudoglandular pattern of differentiation, which may be difficult to discriminate from CCC. CCC may also demonstrate a trabecular architecture, overlapping with the low power histology of HCC. Histologically, HCC with pseudoglands will lack mucin and will often contain bile plugs within the pseudo lumen. p-CEA will delineate the canalicular pattern of staining in HCC. HepPar-1 is also a useful IHC tool, which

will be positive in HCC and negative in CCC in the majority of cases. The tumor cells in CCC will be immunoreactive for cytoplasmic p-CEA, CA19, EMA, and BER-EP4. Cytokeratin panels are not especially helpful in differentiating HCC from CCC.

Other differentials include benign lesions such as bile duct hamartoma and peribiliary gland hamartoma (bile duct adenoma). These lesions are generally small and are found incidentally. Cytologically, the lesions lack the nuclear atypia of cholangiocarcinoma, such as pleomorphism, hyperchromasia, irregular nuclear membranes, and mitoses. Finally, epithelioid Hemangioendothelioma contains vascular spaces that may mimic glandular lumina on H& Eexamination. If in question, mucicarmine and IHC stains such as CD34 and CD31 will resolve this differential [51].

1.2.7. ***Treatment, Outcome and Prognostic factors:***

CCC carries a poor prognosis, with a median survival from diagnosis of approximately 6 months [51]. Chemotherapy and radiation treatment have not shown great efficacy. Surgical resection and transplantation are the only hope of a cure; however, 80% of patients present with unresectable tumors [147]. The most frequent sites of metastasis include lymphnodes, lung, peritoneum, adrenal gland, kidney, and bone [80]. In patients with resectable disease, the median survival rate is 12 to 23 months [144, 147-150]. As would be expected, tumors with lymphatic or perineural invasion, sarcomatoid change, larger tumor size, tumor necrosis, positive surgical margins, and lymph node metastases are associated with a poorer prognosis [51].

1.3. Fibrolamellar Carcinoma:

Although fibrolamellar carcinoma (FLC) has conventionally been considered to be a histologic variant of hepatocellular carcinoma, it has more recently been recognized as a distinct clinical entity with respect to its epidemiology, etiology, and prognosis.

1.3.1. CLINICAL FEATURES:

Fibrolamellar carcinoma is recognized by characteristic histologic changes,

prolonged survival relative to conventional HCC, and clinical appearance in adolescents or young adults [51]. The tumor as a distinct entity has been recognized for many years, and has been reported as eosinophilic HCC with lamellar fibrosis, polygonal cell type HCC with fibrous stroma, and fibrolamellar oncocytic hepatoma [51, 151-154]. It is more common in Western countries and relatively rare in Asia and Africa [143]. The vast majority of tumors of this variant arise in noncirrhotic livers and are not associated with factors implicated in the development of other hepatic tumors, such as hepatitis B or C virus infection, alcohol consumption, or use of oral contraceptives. A recent retrospective cohort study using SEER program found that FLC comprised less than 1% of all primary liver malignancies and 13.4% of all cases in patients younger than 40 years of age (U.S. data) [155]. The mean age of patients with FLC was 39 years versus a mean age of 65 years in patients with HCC. Although previous studies have shown that males and females are equally affected, in this study FLC was more common in women. Increased patient survival was confirmed in this study, with a 5-year relative survival rate of 31.8% versus 6.8% for HCC. Although FLC is more commonly found in younger patients, the most common malignant liver tumor in children and young adults is still conventional HCC [156]. FLC generally comes to clinical attention because of abdominal pain, malaise, fatigue, hepatomegaly, or epigastric mass [156, 157]. Rare reported presentations of FLC include Budd-Chiari syndrome, anemia, hypoglycemia, obstructive jaundice, and gynecomastia [156, 158-165]. Serum aminotransferases are often normal or only mildly elevated [158]. Serum AFP levels may be mildly elevated in a minority of cases (between 5% and 15%, with levels usually below 100 ng/mL) [152, 166]. A more helpful serum marker is DCP, which has been reported to be consistently elevated in FLC [167]. Previously investigated serum markers include vitamin B12, unsaturated B12 binding capacity, and neurotensin, but these studies have shown mixed results [167-170]. Clinically, the differential diagnosis of FLC can be quite difficult. On imaging, the central scar of FLC may mimic FNH or large hepatic hemangioma. The presence or absence of calcification may aid in the radiologic diagnosis; calcification is much more common in FLC than FNH [171]. Additional features that help to distinguish FLC from other liver lesions with central scars include tumor size larger than 10 cm, width of tumor scars, presence of surface

lobulation, vascular invasion, and lymphadenopathy [172]. The pathogenesis of FLC remains a mystery. The common presence of a central scar in FNH and FLC has previously led to the speculation that they share a common pathway of development. However, only rarely have the two tumors been reported in association [173, 174]. Recent studies into the molecular background of FLC have revealed overexpression of genes in the *RAS*, MAPK, *PIK3*, and xenobiotic degradation pathways [175]. FLC is not associated with the risk factors for HCC, and does not share the genetic abnormalities that have been demonstrated in conventional HCC, such as p53 mutation, survivin overexpression, β-Catenin mutation, microdeletions of p14ARF, increased Mdm2 expression, gankyrin overexpression, repression of p16, and loss of heterozygosity in the insulin-like growth factor 2 receptor (IGF2R) locus [57, 59, 156, 176, 177].

1.3.2. PATHOLOGIC FINDINGS:

1.3.2.1. Macroscopic features:

FLC is most often found as a solitary mass (80% to 90%) involving the left lobe of the liver [51]. The tumor is grossly well circumscribed, multinodular, firm, yellow-tan or brown, with a bulging cut surface, and may show focal bile staining. The most distinctive and defining gross feature of FLC is the presence of a central scar with radiating fibrous septae. Necrosis and hemorrhage are frequently present. The tumor is large at presentation, ranging in average size from 9 to 14 cm in greatest dimension [156]. The background liver is noncirrhotic in the vast majority of FLC cases. A handful of case reports describe an association of nodular regenerative hyperplasia or focal nodular hyperplasia with FLC [173, 174]. Lymph node metastases are common at presentation [178].

1.3.2.2. Microscopic features:

The tumor is composed of sheets, nests, and trabeculae of cells with intervening hyalinized, relatively acellular, collagenous parallel bands (‘‘fibrolamellar’’), which is characteristic of FLC. An adenoid or pelioid architecture may also be seen. The tumor cells are large and polygonal, with

well-defined cell borders and eosinophilic, coarsely granular cytoplasm [143]. The nucleus is large and vesicular with a prominent macronucleolus; mild nuclear pleomorphism may be seen. Although mitoses are rare, areas of necrosis and vascular invasion are occasionally identified. The tumor often contains small, thick-walled arteries. Stainable copper and bile may be demonstrated in the cytoplasm of most tumors. Eosinophilic hyaline globules, which are often PAS- and PAS-DR (diastase-resistant)–positive, are present in approximately half of cases. As in other primary liver tumors, steatosis and Mallory hyaline may be present. Although rare, intracytoplasmic mucin may be seen in a combined form of HCC-cholangiocarcinoma. So called cytoplasmic pale bodies, or ground-glass inclusions, are often seen in FLC. These inclusions contain fibrinogen, which is highlighted by immunoperoxidase staining. On ultrastructural examination, pale bodies are associated with intracytoplasmic lumina/ bile canaliculi or accumulation of rough endoplasmic reticulum [179]. Fine needle aspirate smears demonstrate the tumor cells singly, in small groups, or in sheets. The tumor cells are often larger than benign hepatocytes or the cells of conventional HCC, with abundant granular eosinophilic cytoplasm. Strands of collagen are sometimes identified. Many of the features of conventional HCC in smear preparations, such as thickened trabeculae and thin, arborizing vascular channels, are not seen in FLC [156, 180]. By electron microscopy, the cytoplasm contains numerous mitochondria, creating the eosinophilic, coarsely granular appearance by light microscopy (oncocyte). So-called dense core neuroendocrine-like granules have been reported [181]. IHC findings in FLC reflect the hepatocyte differentiation of the tumor. HepPar-1 is strongly positive in the majority of tumor cells. A variety of cytokeratin expression is seen, including strong positivity for CK8 and CK18, as well as CK7 and CK19, which are normally expressed in biliary epithelium. FLC is more likely to be positive for CK7 than is conventional HCC. Expression of neuroendocrine markers may be present. Matrix metalloproteinase 2 expression has more recently been recognized in the tumor cells [182]. A1AT, alpha-fetoprotein, fibrinogen, and C-reactive protein have all been variably demonstrated in FLC [154, 156, 183, 184]. Variants of the ‘‘classic’’ FLC have been recognized. These include clear-cell FLC and pseudoglandular FLC. Clear-cell FLC has been reported only twice in the literature, and is described

as clear cells and oncocytic cells arranged in trabeculae, separated by prominent lamellar fibrosis [185, 186]. On ultrastructural examination, the clear cells contain numerous ballooned mitochondria. The tumor is otherwise similar to FLC and the behavior is unknown. Pseudoglandular FLC, also described as combined FLC and cholangiocarcinoma, demonstrates areas of gland-like (pseudogland) formation composed of cells resembling the rest of the tumor. Mucin formation can be demonstrated by mucicarmine stain. Other reports describe FLC containing gland formation resembling biliary epithelium, which stain diffusely for HepPar-1 and behave similarly to conventional FLC [187]. As in clear-cell FLC, the reported number of cases is too small to draw significant conclusions as to behavior and prognosis. FLC rarely contains areas histologically consistent with usual HCC [188]. Similarly, cases have been reported in which FLC and HCC are reported as two separate, synchronous tumors [189]. The histologic pattern of metastatic FLC is generally similar to the primary tumor; however, cases have been reported in which the metastasis demonstrates features of typical HCC [190]. The behavior of these ‘‘mixed’’ tumors is unknown at this point due to the small sampling of cases.

1.3.3. DIFFERENTIAL DIAGNOSIS:

The differential diagnosis of FLC includes tumors with a central scar or significant fibrous reaction, such as FNH, scirrhous HCC, cholangiocarcinoma, and adenosquamous or squamous cell carcinoma with a fibrous reaction. FNH lacks the cytologic atypia of FLC. HCC may show fibrosis (scirrhous HCC), or have oncocytic features. Grossly, scirrhous HCC shows a more diffuse pattern of fibrosis; microscopically, FLC is characterized by much larger cells, demonstrates more prominent nucleoli, and lacks the abundant thickened trabeculae of HCC. Although FLC may show pseudoglandular formation, cholangiocarcinoma histologically will lack the defining features of FLC. Metastatic tumors with a prominent desmoplastic response, such as pancreatic adenocarcinoma or squamous carcinoma, may also be considered in the differential diagnosis. These tumors are easily discriminated histologically: pancreatic adenocarcinoma is characterized by gland formation, and squamous cell carcinoma will show keratinization and intracellular bridging. Neuroendocrine tumors are also an important consideration; these tumors may

have abundant cytoplasm, a branching fibrous stroma, and an overlapping IHC pattern. However, they lack the lamellar pattern of fibrosis and demonstrate an organoid or serpiginous pattern, which is not present in FLC.

1.3.4. TREATMENT AND OUTCOME:

FLC has been demonstrated to have an overall better prognosis relative to HCC. However, this may be due in part to the younger age of the patients and the lack of background cirrhosis. As in many other tumors, resectability is the most important prognostic factor for FLC, with a reported median 5-year survival of 76% with resectable disease versus 0% with unresectable disease [188]. Approximately one third of patients with resectable disease have lymphovascular invasion and 50% show lymph node metastases at the time of surgery [188]. Tumors also commonly metastasize to peritoneum and lung.
Treatment options include partial hepatectomy and transplantation. In one series, overall survival following surgical intervention was 89.5% (1 year), 75% (3 years), and 50% (5 years) [191]. Chemotherapy options are available for treatment of FLC, including fluorouracil (FU) and recombinant interferon alfa-2b (rIFNalpha2b), with reported survival benefits [192].

1.4. Combined Hepatocellular Carcinoma-Cholangiocarcinoma:

Combined hepatocellular carcinoma/ cholangiocarcinoma (HCC-CCC) is a rare tumor, representing less than 1% of all primary liver carcinomas [51, 143]. The tumor contains elements of both HCC and CCC admixed within the same tumor mass (as distinguished from two separate tumors of HCC and CCC within the same liver). The geographic distribution, patient age and sex, hepatitis virus infection status, and levels of serum AFP are more similar to conventional HCC than to CCC [143, 193]. Combined HCC-CCC exhibits a slightly more aggressive behavior than pure HCC or CCC. A recent study demonstrated that combined tumors show more invasion into the portal vein than HCC or CCC [190]. The frequency of lymph node metastases has been reported to be slightly higher in HCC-CCC than in HCC [143], but is significantly less than in pure CCC [193].
Macroscopically, tumors with a significant component of CCC will show a

prominent white, firm, fibrous stroma [143]. Goodman et al described three histologic types in 1985, including collision tumors, transitional tumors, and fibrolamellar tumors [194]. Collision tumors represent a coincidental occurrence of both HCC and CCC in the same patient. Transitional tumors contain areas of intermediate differentiation and a recognizable transition between HCC and CCC. Fibrolamellar tumors describe FL-HCC with areas of pseudoglandular formation. The current (2000) WHO classification of tumors considers the defining feature of HCC-CCC to be the presence of distinct and separate HCC and CCC elements within the same tumor mass [143]. Collision and fibrolamellar tumors should not be considered combined HCC-CCC under the current definition. Conventional HCC may demonstrate prominent pseudoglandular spaces, and this should not be confused with combined HCC-CCC. The pseudoglandular formation may be recognized by the similarity of the lining cells to hepatocytes, and the presence of bile rather than mucin [51]. Metastases of combined HCC-CCC may show a similar histologic appearance or may show elements of either HCC or CCC alone. The neoplastic hepatocytes are positive for CK8 and CK18, as in conventional HCC, as well as CK7 and CK19 (markers of biliary epithelium). The most useful diagnostic markers are p-CEA (highlights the bile canaliculi) and HepPar-1 (cytoplasmic) in the HCC component, and diastase-resistant PAS positivity demonstrating the neutral epithelial mucin in the CCC component [143]. The prognosis of HCC-CCC has been reported to be worse relative to conventional HCC [143]. The overall 5-year survival rate reported in a recent series was 23.1% for combined HCC-CCC, as compared to 66.2% for HCC and 32.3% for CCC [193]. Predictors of poor outcome included macroscopic vascular invasion and bilobar tumor.

1.5. Active Signaling pathways in primary liver cancer:

1.5.1. Wnt/β-Catenin pathway:

The Wnt/β-Catenin pathway is a well-conserved pathway that is important in embryonic development, cell proliferation, survival, regeneration and self-renewal [195-197].

Based on the earlier studies, the Wnt/β-Catenin pathway is a central player in maintaining liver health and is dysregulated in hepatic cancers, which makes it an attractive candidate for potential therapies of HCC.

In an unstimulated cell, endogenous β-Catenin is found at the adherens junctions, where it interacts with components of the cadherin-associated protein complexes to confer cell-cell adhesion functions [198, 199]. On the other hand, surplus β-Catenin in the cytoplasm is degraded by the action of a destruction complex which consists of glycogen synthase kinase 3β (GSK3β), Axin, adenomatous polyposis coli (APC) and casein kinase Iα (CKIα) [200]. β-Catenin is first phosphorylated at serine-45 (Ser45) by CKIα to further prime it for phosphorylation by GSK3β at Ser33, Ser37 and threonine-41 (Thr41). The phosphorylated β-Catenin is then ubiquitinated by β-transducin repeat-containing protein (β-TrCP) and subsequently degraded by the proteasome [201] (Figure 3A).

Maher et al [202] reported that β-Catenin phosphorylated at Ser45 and not at Ser33/Ser37/Thr41 is predominantly located in the nucleus, whereas β-Catenin phosphorylated at Ser33/Ser37/Thr41 is mostly localized to the cytoplasm.

For diseased condition, the Wnt/β-Catenin signalling pathway is activated upon binding of Wnt to one of the members of the frizzled (FZD) family and to low density lipoprotein receptor-related protein 5 or 6 (LRP5/LRP6). The FZD recruits dishevelled (Dvl) to the plasma membrane, which in turn recruits Axin and GSK3β to LRP5/LRP6 [203]. The intercellular domain of LRP5/LRP6 contains five reiterated PPPSPxS motifs, which are dually phosphorylated by GSK3β and CKIα [204]. The phosphorylation of LRP5/LRP6 disrupts the formation of the destruction complex, thereby preventing GSK3β from phosphorylating β-Catenin. Therefore, β-Catenin is not degraded and accumulates in the cytoplasm from where it translocates to the nucleus.

In the absence of Wnt, T-cell factor (TCF)/lymphoid enhancer factor (LEF) represses gene expression by interacting with co-repressor Groucho, which promotes histone deacetylation and chromatin modelling in the nucleus [205]. Nuclear accumulation of β-Catenin displaces Groucho from TCF/LEF and recruits other transcriptional co-activators, e.g. CREB binding protein (CBP), for upregulation of target genes that are implicated in cell proliferation, anti-

apoptosis, and angiogenesis, such as cyclin D1 (and c-myc, and MMP-7, and thus contribute to carcinogenesis) [206] (Figure 3B).

1.5.2. MAP kinase Pathway:

Mitogen-activated protein kinases also known as MAP kinases are serine/threonine-specific protein kinases belonging to the CMGC (CDK/MAPK/GSK3/CLK) kinase group. MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines. They regulate proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis - among many others.

The pathway comprises a multistep phosphorylation cascade composed of a downstream network of protein kinases. The activation of the pathway occurs normally through a series of receptors tyrosin kinase (RTKs) (EGFR, HER2, KIT, PDGFRA), which reacts physiologically of the bindings of ligands leading to autophosphorylation (binding of phosphate groups on the amino acid Tyrosin in the intracellular part of the receptor). This activates indirectly the *RAS*, which in turn leads to the recruitment and phosphorylation of *RAF*. *RAF* activates MEK which also leads to the activation and phosphorylation of ERK. ERK is responsible for the regulation of different proteins such as transcription factors, cell cycle proteins and antiapoptotic factors (Figure 4). That can result in a wide spectrum of effects within the cell which vary depending on the cell type [207].

The activation of the MAPK signal way in the tumor cells occurs through mutations in different genes [208-210], of which the so called “driver mutations” play the main role in the development and preservation of the malignant phenotype.

BRAF is one of three *RAF*-Genes (A, B and C*RAF*) in the MAPK signal way. Activating mutations of *BRAF* are found most frequently in the P-Loop (exon 11) and the activated segment (exon 15) of the kinase domain. The most frequent mutation (about 90% of all cases) is V600E [210]. Somatic *BRAF* mutations present in about 8% of human tumors especially in thyroid carcinoma and colon carcinoma. Also 100% of the hairy cell leukemias and >50% of Langerhans cell histiocytosis cases show *BRAF* mutations. Except for few exceptions, *BRAF* and *RAS* mutations seem to be mutually exclusive so that one must suppose

that both genetic changes activate the same effectors of transformation [210, 211].

Vemurafenib is a *BRAF* inhibitor which introduced in 2011 for the therapy of metastasized melanomas harbouring *BRAF* mutations leading to a significant survival benefit [212].

The introduction of new medications for the *BRAF* targeted therapy (and other substances which block the downstream kinase MEK) goes forward with very wide steps which should actually trigger the oncology oriented research to find further tumors in which the *BRAF* mutations play at least a partial role in the tumorogenesis.

1.6. Goal of the study:

In this article, we aimed at describing novel immunohistochemical markers for detecting primary liver malignancies, and identifying new molecular biomarkers as potential contributors to hepatocarcinogenesis.

2. Material and Methods:

2.1. The clinicopathological and immunohistochemical analysis:

2.1.1. Study population:

HCCs and CCCs cases were retrieved from the files at Institute of Pathology, Eberhard Karls University in Tübingen.
A total of 51 HCCs between 2003 and 2010, 47 CCCs between 2000 and 2011 (including 40 ICC and 7 hilar cases), 3 cases of combined HCC-CCC and 8 cases of FLC (from 7 patients as in one case both the original tumor -2005- and the recurrent tumor -2008- were sampled) were identified of which cell blocks were available.
The H&E sections of the cases were reviewed and found to be qualified (after confirmation of the original diagnosis). Cases were graded as: well, moderately, and poorly differentiated according to the World Health Organization classification and staged based on the American Joint Committee on Cancer/Union Internationale Contre le Cancer pTNM system 2010.

Pathologic features analyzed included tumor size and multiplicity (presence of two or more tumor nodules), lymph node status and status of the margins of resection; which was determined on the basis of the gross description recorded in the pathology report. In addition the H&E sections of the cases were reviewed regarding presence of lymphatic or vascular invasion and other special features such as: nuclear grade, histologic patterns and cytologic variants, presence or absence of inclusions and steatosis and presence or absence of cirrhosis in the background liver.
Clinical data and follow-up information were obtained from patients' charts.

2.1.2. Construction of the Tissue Micro Arrays:

Formalin-fixed, paraffin-embedded samples were used to build a set of four Tissue Micro Arrays (TMA) corresponding to HCC, CCC, combined HCC/CCC and FLC following a previously described protocol [213]. Tumors and paired nonneoplastic liver were spotted 3 to 3 times each, using 1-mm cores.

2.1.3. Selection of the antibodies:

A wide variety of antibodies were selected starting from standard ones (HepPar1, Glypican3, CD10, AFP, β-Catenin, Wnt1, CK7, P53 and MIB1), to the protein candidates from the IndividuaLIVER project (i.e. FGG, Claudin 10, DEK, APOL1, HSP90, MMP7, APOB, ABCC3 and eEF2), to SALL4 as a stem cell marker, *BRAF* because of the scarce and contradictory reports regarding *BRAF* in the literature.

IndividuaLIVER project is a multidisciplinary study performed at Tübingen University on the Immunotherapy of primary liver malignancies. The study identified recurrent genomic and phenotypical alterations in HCC and CCC, and built a list of markers (HLA-attached peptides or genes which are partners in a putative fusion transcript) for the screening of patients with these malignancies.

In spite of applying all the above markers to the TMA sections, we investigated in detail only the results of the standard diagnostic markers and the novel ones (SALL4 and *BRAF*), in addition to FGG from the IndividuaLiver study because of its unique type of expression.

The antibodies used are summarized in the following table:

Antigen	Clone	Company	dilution	Secondary Antibody	Type
SALL4 (M03)[a.]	6E3	Abnova	1:500	Mouse. M, isotype: IgG1 Kappa	n
B-Catenin	CAT-5H10	Zytomed	1:400	Rabbit. P, isotype: IgG	n, m, c[f.]
BRAF [b.]	VE1	Spring Bioscience	1:50	Mouse. M, isotype: IgG2a	c
P53 [c.]	DO-7	Novocastra	1:200	Mouse. M, isotype: IgG2b	n
Ki-67 [d.]	MIB-1	Dako	1:200	Mouse.M, isotype: IgG1 Kappa	n
FGG [e.]	EPR3084	RabMAbs	1:50	Rabbit. M, isotype: IgG	c
HepPar1	OCH1E5	Dako	1:500	Mouse. M, isotype: IgG1 Kappa	c
Glypican3	HPA006316	Sigma-Aldrich	1:400	Rabbit. P, isotype: IgG	c
AFP	A0008	Dako	1:1000	Rabbit. P	c
CK7	OV-TL 12/30	Dako	1:1000	Mouse. M, isotype: IgG1 Kappa	m
CD10	56C6	Novocastra	1:30	Mouse. M, isotype: IgG1	m
Wnt1	DRAQ7	Abcam	1:100	Rabbit. P, isotype: IgG	n

a. antibody reactive against recombinant protein.

b. peptide representing the *BRAF* V600E mutated amino acid sequence from amino acid 596 to 606.

c. recombinant human wild type P53 protein.

d. Human recombinant peptide corresponding to a 1002 bp Ki-67 cDNA fragment.

e. Synthetic peptide corresponding to residues in Human Fibrinogen gamma chain.

f. β-Catenin is typically expressed in the cell membrane and cytoplasm, whereas the nuclear expression is aberrant.

M= monoclonal, P= polyclonal, n= nuclear positivity, c= cytoplasmic positivity, m= membranous positivity.

2.1.4. Immunohistochemistry procedure:

Immunostaining was performed using BenchMark XT IHC/ISH autostainer (Ventana Medical Systems, Roche; Tucson, Arizona). Briefly, 2.5μm sections were deparaffinised and subjected to heat-induced antigen retrieval using EDTA buffer (pH 8.4) for 64 minutes (standard CC1).
Prolonged antigen retrieval (74 min with Ultraview) was used with the preparations to be stained with FGG. Subsequently, incubation with the primary antibody (in the 37° temperature for 32 min) was followed by detection of reaction using iVIEW DAB (diamino benzidine hydrochloride) v3 kit. All slides were counterstained with hematoxylin (4 min incubation).

2.1.5. Evaluation of immunostains:

The immunoreactivity was estimated according to two parameters:
1-The number of tumor cells positive for the staining (focal staining versus diffuse) was graded for the SALL4 stain as: 0 when no positive cells identified; 1+ when <25% of tumor cells are immunoreactive for the stain; 2+ when 25-50% of tumor cells are immunoreactive; 3+ when 50-75% of tumor cells are positive for the stain and 4+ when more than 75% positive cells are observed,
for the P53 stain as: 0 when no positive cells identified; 1+ when <10% of tumor cells are immunoreactive for the stain; 2+ when 10-19% of tumor cells are immunoreactive; 3+ when 20-49% of tumor cells are positive for the stain and 4+ when more than 50% positive cells are observed,

and for the MIB1 stain as: 0 when no positive cells identified; 1+ when <5% of tumor cells are immunoreactive for the stain; 2+ when 5-9% of tumor cells are immunoreactive; 3+ when 10-19% of tumor cells are positive for the stain, 4+ when 20-49% of the cells positive and 5+ when more than 50% positive cells observed.

Evaluation of the immunohistochemical stains according to the number of positive cells:

	0	**1**	**2**	**3**	**4**	**5**
SALL4	no positivity	<25%	25-50%	50-75%	>75%	
P53	no positivity	<10%	10-19%	20-49%	>50%	
MIB1	no positivity	<5%	5-9%	10-19%	20-49%	>50%

2-The intensity of the staining (weak staining versus intense) ranged from 1+ for weak staining; 2+ for intermediate degree and 3+ for intense staining.
Accordingly the results appear as a number which is the result of multiplying the two above mentioned values.

The site of positivity was evaluated in the case of β-Catenin stain (cytoplasmic/membranous versus aberrant nuclear) in addition to the number of tumor cells positive for the stain (focal versus diffuse).
The presence of canalicular positivity was evaluated in the case of CD10.

2.2. The molecular analysis:

We also applied the polymerase chain reaction to detect *BRAF* V600E hotspot mutations using the Sanger Sequencing method and allele specific PCR with melting point analysis.

2.2.1. **DNA extraction and *BRAF* mutation detection (Sanger Sequencing)**

DNA used for PCR was extracted from 10 µm paraffin sections after dewaxing and proteinase K digestion applying standard phenol/chloroform purification procedures [214]. DNA was amplified for exon 15 of *BRAF* including codon 600 using the following primers:
5'-TCATAATGCTTGCTCTGATAGGA-3'

and 5'-CTAGTAACTCAGCAGCATCTC-3'. PCR was performed using 200 ng DNA template in a final volume of 50 µl with 0.2 µM of each primer, 0,2 mMdNTPs, 1.5 mM MgCl2 and 1,5 Units Taq polymerase (AmpliTaq Gold® DNA Polymerase, Applied Biosystems, Foster City, CA). Cycling conditions entailed an initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation (95°C for 45 sec), annealing (56°C for 45 sec) and elongation (72°C for 60 sec), with a final elongation at 72°C for 7 min.

PCR products were purified (AMPure, Beckman Coulter, Brea, CA, USA) and aliquots of 7 µl were used for the sequencing reaction with 1 µM of the universal sequencing primer and 2 µl of GenomeLab DTCS-Quick Start Kit (Beckman Coulter, Brea, CA, USA) in a final volume of 10 µl according to the manufacturers protocol. Sequencing reactions were purified (CleanSEQ, Beckman Coulter, Brea, CA, USA) and analyzed in a GenomeLabGeXP Genetic Analysis System and evaluated by the GenomeLabGeXP software 10.2 (Beckman Coulter, Brea, CA, USA) to determine the mutation status.

2.2.2. Molecular Detection of *BRAF* V600E mutation (Melting point analysis)

After macrodissection of paraffin-embedded tissue, dewaxing and digestion with proteinase K for 16 hours, genomic DNA was purified applying standard phenol/chloroform purification [214]. Detection of the *BRAF* V600E (c.1799T>A) mutation was performed by melting curve analysis of amplification products with the Light Cycler System (Roche Applied Science, Mannheim, Germany) [215]. The primers (forward: 5'-TCATAATGCTTGCTCTGATAGGA-3' and reverse: 5'-GGCCAAAAATTTAATCAGTGGA-3') were used to amplify the region around codon 600. Amplicons to be genotyped by melting point analysis were separately generated by conventional PCR and by peptide locked nucleic acid (LNA)-mediated PCR clamping with a wild-type specific LNA oligonucleotide (5'-TAGCTACAGTGAAATCTC-PH -3'). PCR amplifications were performed in final reaction volumes of 50 µl, containing 2.5 mM $MgCl_2$, 0.2 mMdNTPs, 0.2 µM primers and 1.5 Units Taq polymerase (AmpliTaq Gold® DNA Polymerase) with 0.04 µM LNA in the respective reaction. Each of the 50 PCR cycles consisted of denaturation at 95°C for 45 sec, annealing at 56°C for 75 sec and extension at

72°C for 45 sec. The first cycle was preceded by denaturation at 95°C for 60 sec and the last cycle was extended by a 7 min elongation step at 72°C. Subsequent melting point analysis was performed using 10 µl PCR product with 0.05 µM anchor and sensor probe and 2 mM $MgCl_2$.

2.3. Statistical analysis:

Chi-square or Wilcoxon rank-sum test was used to evaluate possible associations between covariates (IHC-expression, mutated and wild-type genotypes) and clinical or pathological parameters. Statistical comparisons were performed using a statistical software package. P-values of less than 0.05 were considered to be statistically significant.

3. ***Results (Table 6A und 6B):***

3.1. ***Clinical and pathologic findings:***

The clinicopathologic features of all cases are summarized in Table 7.

3.1.1. **Of the HCC cases**; forty five patients (88.2%) in this series were males. They ranged in age from 16 to 77 years (mean, 64.6 years).
Tumor size ranged from 1.4 to 21 cm (mean, 6.2 cm).
The background liver shows a precirrhotic or cirrhotic architecture in 29 cases (56.9%). The cirrhosis was associated with viral genesis in 10 (19.6%) cases (4 with HBV and 6 with HCV), with alcohol abuse in only 2 cases (3.9%) and with metabolic disorders in 3 (5.9%) cases (2 cases with Hemochromatosis and one case with Wilson disease). The rest cases were marked as cryptogenic cirrhosis.
Topographically, there have been 17 cases (33.3%) in the right liver lobe (segments 5-8), 13 cases (25.5%) in the left one (including the caudate/ segment 1, the lateral/ segments 2, 3 and medial/ segments 4a, 4b lobes), whereas 15 cases (29.4%) show involvement of both hepatic lobs. In 6 cases (11.7%) was no topographic placement possible (these are liver explantations with small tumors -T1- without referring to the tumor location in the macroscopic description).
In the setting of determining histologic grade; 42 HCCs (82.4%) are grade 2 (moderately differentiated), 8 (15.7 %) are grade 1 (good differentiated) whereas only one case (1.9%) is grade 3 (poorly differentiated). No undifferentiated (grade 4) HCC was diagnosed. Only two cases had two histologic grades in one tumor (grade 1 and 2, in which the highest grade was considered).
Regarding the histologic subtypes and the cytologic variants; the trabecular subtype is evident in 32 (62.7%) cases (pure trabecular in 24 cases or with areas of solid growth pattern in 8 cases), the pseudoglandular subtype in 16 (31.4%) cases (at least as partial differentiation admixed with trabecular or solid areas in 14 cases, rarely as a pure pseudoglandular pattern -one case- or as a second tumor nodule with pure pseudoglandular differentiation in one case too;

patient no. 13, Table 7), whereas the pure solid subtype is observed only in 3 case (5.9%). Figure 5 illustrates some of the pathologic variants detected in our cases.

In addition; one case shows partial spindle-cell differentiation (spindle-cell variant) and glycogen and fat accumulation is observed at least as a focal feature in 17 cases (clear-cell variant). No cases of pleomorphic- cell variant were observed.

Eleven cases (21.6%) have undergone total hepatectomy with liver transplantation whereas the rest 40 cases (78.4%) underwent partial hepatectomy or lobectomy.

Forty four operations (86.3%) have tumor free margins, whereas 7 operations (13.7%) show infiltrated margins of resection.

Only 13 operations (25.5%) have a lymph node resection, all of which show an N0 status. The number of resected lymph nodes ranged from 1 to 13 (mean, 2.8/resection).

Only 4 cases (7.8%) show lymphatic invasion (L1) which was accompanied in all 4 of them with vascular invasion (V1), whereas 12 cases (23.5%) show vascular invasion (including large vessels invasion) pure or accompanied with lymphatic invasion.

In regards to the tumor stage; 22 cases (43.2%) and 15 cases (29.4%) present in T1 and T2 stages respectively, whereas 12 cases (23.5%) present as T3 (5 cases as T3a and 7 as T3b) and 2 (3.9%) as T4.

3.1.2. We studied the 40 **intrahepatic CCs** further. Of these cases; 18 patients (45%) are males. They ranged in age from 42 to 78 years (mean, 60.5 years).

Tumor size ranged from 0.7 to 12 cm (mean, 6.6 cm).

The background liver shows a cirrhotic architecture only in 4 cases (10%), whilst liver steatosis (with or without the full histologic picture of steatohepatitis) is observed in 15 cases (37.5%). The cirrhosis was associated with HBV infection in two of the four cases. Other special histological features observed are mild to moderate portal fibrosis (in 7 cases or 17.5%) and chronic cholangitis in one case (2.5%).

Topographically, there have been 6 cases (15%) in the right liver lobe, 11 cases (27.5%) in the left one, whereas 21 cases (52.5%) show involvement of both

hepatic lobs. Two cases are atypical resections without referring to the tumor location.

In the setting of histologic grade; 28 ICCs (70%) show the nuclear grade 2, 11 (27.5%) the grade 3, whereas only one case (2.5%) has nuclear grade 1.

Regarding the histologic subtypes; 2 cases of mucinous carcinoma (5%), 2 cases (5%) of signet ring carcinoma (at least as a focal feature) and one case with clear cell features (2.5%) are observed. All other cases (35 or 87.5%) show conventional CC (tubular/ trabecular formations with solid areas or carcinoma with tubular formations within a desmoplastic stroma).

Of the 39 cases which have resections all (100%) have undergone a partial hepatectomy/ hemihepatectomy, the tumor was unresectable in the 40th case and the patient underwent only exploratory Laparotomy with lymphadenectomy (case no. 72, Table 7). Of these cases 16 (41%) have infiltrated margins of resection and 18 cases (46.2%) have free margins. In 5 cases (12.8%) the margins status could not be evaluated (Rx).

Twenty six operations have lymph node resections, 10 of which (38.5%) with N0 status and 16 (61.5%) with N1 status. The number of resected lymph nodes ranged from 1 to 20 (mean, 7.8 nodes/ resection). The number of positive lymph nodes resected ranged from 1 to 9 (mean, 2.5 positive nodes).

Six cases (15.4%) show lymphatic invasion (L1) which was accompanied in 3 of them with vascular invasion (V1), whereas 5 cases (12.8%) show vascular invasion (including large vessels invasion) pure or accompanied with lymphatic invasion.

In regards to the tumor stage; 21 cases (53.8%) present in T1 stage, 13 cases (33.3%) in T2 stage (3 cases as T2a and 10 as T2b), whereas 4 cases (10.3%) present as T3 and only one cases (2.6%) as T4.

3.1.3. Of the 7 **Klatskin cases**; three patients (42.9%) are males whereas 4 (57.1%) are females. They ranged in age from 46 to 69 years (mean, 58.6 years).

Tumor size ranged from 1.5 to 10 cm (mean, 3.9 cm).

The background liver shows no cirrhotic architecture in all cases (100%). A mild to moderate portal fibrosis is evident only in one case.

Topographically, there have been 5 cases (71.4%) in the left hepatic lobe whereas 2 cases (28.6%) show involvement of both hepatic lobs. No cases involving the right hepatic lobe identified.
In the setting of histologic grade; 5 cases (71.4%) show the nuclear grade 2 whereas the nuclear grade 1 and 3 are evident in one case each.
Regarding the histologic subtypes; all the cases show the conventional CCC morphology which is accompanied with focal signet ring features only in one case (14.3%).
All the cases have undergone a partial hepatectomy/ hemihepatectomy, of which 4 cases (57.1%) have infiltrated margins of resection and 2 cases (28.6%) have free margins. In one case (14.3%) the margins status could not be evaluated (Rx).
Six operations have lymph node resections, 3 of which (50%) show an N0 status and the other three an N1 status (50%). The number of resected lymph nodes ranged from 4 to 25 (mean, 13.8 nodes/ resection). The number of positive lymph nodes resected ranged from 2 to 8 (mean, 4.3 positive nodes).
Only one case (14.3%) shows lymphatic invasion (L1) which was accompanied with vascular invasion (V1), whereas 4 cases (57.1%) show vascular invasion (including large vessels invasion) pure or accompanied with lymphatic invasion.
In regards to the tumor stage; 4 cases (57.1%) present in T2 stage (2 cases as T2a and 2 as T2b), whereas 2 cases (28.6%) present as T3 and only one case (14.3%) as T4. No cases present as T1 (0%).

3.1.4. Of the **HCC/CCC** cases, two present in male-patients and one in female-patient. They ranged in age from 14 to 66 years (mean, 42 years).
Tumor size ranged from 1.8 to 7 cm (mean, 5.1 cm).
The background liver shows cirrhotic/ precirrhotic architecture in all cases.
Topographically, there have been 2 cases in the right hepatic lobe whereas one case involved both hepatic lobs.
In the setting of histologic grade; 2 cases show the nuclear grade 3 whereas the third case is of grade 2.
All the three cases have undergone a partial hepatectomy/ hemihepatectomy with free margins of resection (R0).
Two cases have an N0 status and one case an N1 status.

One case shows lymphatic invasion (L1), also one case shows vascular invasion (V1).
In regards to the tumor stage; 2 cases present as T2a whereas the third as T1.

3.1.5. Four of the 7 **FLC** patients are males and three are females. They ranged in age from 17-66 years (mean, 31.4 years). The background liver shows no cirrhotic architecture in all cases. All the cases have the histological grade 2.
Further clinicopathologic information was available only to 4 of the cases. Tumor size ranged from 12 to 14.5 cm (mean, 13.6 cm).
All the four cases underwent partial hepatectomy or lobectomy. Three operations have tumor free margins, whereas the fourth one has infiltrated margins of resection.
Three cases have N1 status and one has N0 status. The number of resected lymph nodes ranged from 3 to 11 (mean, 7.5 nodes/ resection). The number of positive lymph nodes resected ranged from 1 to 2 (mean, 1.7 positive nodes).
One case shows lymphatic invasion (L1) and one vascular invasion (V1).
Regarding the tumor stage; one case presents in T1 stage, another one in T3b stage and two cases present as T4.

Noticed in some cases were some special features such as the association of one of the FLC cases with focal nodular hyperplasia FNH (patient no. 103, Table 7).

3.2. ***Immunohistochemistry:***

3.2.1. HCC:

Forty six HCCs (90.2%) are positive for HepPar1. The staining showed focal to diffuse mostly granular but sometimes homogenous cytoplasmic positivity. In the cases with clear cell features the staining was membranous. The normal parenchyma showed moderate to strong cytoplasmic positivity in all cases (100%).
50 HCC cases (98%) are positive for Glypican3 with different patterns of expression which ranged from moderate to strong sometimes granular at sites heterogenous cytoplasmic positivity to typical canalicular positivity which is

evident in 6 cases (11.7%). The normal liver showed moderate to strong granular cytoplasmic positivity in all cases (100%).

4 HCC cases (7.8%) showed focal to diffuse AFP positivity with concurrent moderate positivity of the adjacent normal liver only in one case (1.9%).

10 cases (19.6%) show CK7 positivity. This was typically focal and moderate to strong in 8 cases and diffusely strong in two cases.

4 cases (7.8%) are positive for SALL4 of which one case (1.9%) shows diffuse and strong nuclear positivity and three cases (5.9%) show mild to moderate focal positivity in at least 25% of the tumor cells. No positivity in the normal parenchyma observed (Figure 6).

Five HCCs (9.8%) show focal and weak to moderate FGG positivity (in <25% of the tumor cells).

10 cases (19.6%) show focal nuclear β-Catenin positivity simultaneously with diffuse membranous or cytoplasmic positivity. 41 cases (80.4%) show focal to diffuse membranous positivity. This was accompanied by focal cytoplasmic positivity in 18 cases of them and with diffuse strong positivity in the adjacent parenchyma only in one case.

Diffuse and strong granular cytoplasmic Wnt1 positivity is evident in 48 cases (94.1%). Only three cases show absence of expression of Wnt1. Interestingly all the three cases are positive for SALL4.

None of the HCC cases show immunoreactivity with the *BRAF* antibody.

13 cases (25.5%) show nuclear accumulation of P53 (moderate to strong accumulation in less than 5% of the tumor cells in 10 cases, moderate to strong accumulation in 15% of the cells in two cases and strong accumulation in 90% of the tumor cells in one case).

A mild proliferation activity (as evident in the MIB1 stain) is observed in 39 cases or 76.5% (mild to strong nuclear expression in <5% of the tumor cells in 35 cases and strong expression in 10% of the cells in 4 cases), whereas a moderate proliferation activity (MIB1 positivity in 20-30% of the tumor cells) is evident in 3 cases (5.9%).

36 cases (70.6%) show typical expression of CD10 in a canalicular pattern. The adjacent parenchyma stains appropriately.

3.2.2. CCC:

Of the 40 ICC cases 38 cases (95%) show diffuse and strong cytoplasmic positivity for CK7. The other two cases have only focal positive cells.

Weak to moderate nuclear positivity for SALL4 is observed only in 3 cases (7.5%) as diffuse positivity (in >70% of the tumor cells) in two case and as focal positivity in about 5% of the tumor cells in one case, no positivity in the normal parenchyma noted.

BRAF immunoreactivity is not observed in any of the cases.

Three cases (7.5%) show focal nuclear β-Catenin positivity accompanied by diffuse membranous and cytoplasmic positivity. Nine cases (22.5%) show diffuse membranous positivity whereas 28 cases (70%) show cytoplasmic positivity with membranous enhancement.

The adjacent hepatic parenchyma show membranous immunoreactivity sometimes with focal cytoplasmic positivity in all cases.

34 cases (85%) are immunoreactive for Wnt1, whereas 6 cases are negative for the stain.

34 cases (85%) show nuclear accumulation of P53 (moderate to strong accumulation in less than 5% of the tumor cells in 24 cases, moderate to strong accumulation in 10-20% of the cells in 5, moderate to strong accumulation in 30-50% of the cells in 4 cases and strong accumulation in 90% of the tumor cells in one case).

25 cases (62.5%) have a mild proliferation activity (moderate to strong MIB1 expression in less than 5 % of the tumor cells), 9 cases (22.5%) show moderate expression in 10-20% of the cells, 2 cases (5%) have a proliferation activity in 30% of the tumor cells whereas one case (2.5%) has a strong proliferation rate in 60% of the cells.

3.2.3. Klatskin tumors:

Six cases of the hilar CCs are positive for CK7 (85.7%).

None of the cases show positive reaction for SALL4 or *BRAF*.

All cases have a membranous expression of β-Catenin or membranous expression with focal cytoplasmic positivity. This is also noted in the normal parenchyma. No nuclear positivity observed. All the cases (100%) show also positive reaction for Wnt1.

5 cases (71.4%) show nuclear accumulation of P53 (moderate to strong accumulation in less than 5% of the tumor cells in 4 cases and moderate accumulation in 10% of the cells in one case).
4 cases (57.1%) have a low MIB1 expression (in less than 5% of the tumor cells) and two cases (28.8%) show a mild proliferation rate in about 10% of the cells.

3.2.4. Combined HCC/CCC:

CK7 positivity is evident only in the CC component.
Two cases have focal nuclear SALL4 positivity (typically in a dot like pattern in one of them) in 20% and 40% of the cells.
No immunoreactivity for *BRAF* observed.
The three cases show no nuclear β-Catenin positivity. One case showed reduced membranous expression of β-Catenin (practically only focal membranous positivity).
Wnt1 expression is noted in one case and in the HCC component of another one. No expression is observed in the CCC component of this case and in the third one.
One of the cases has a strong nuclear accumulation of P53 in 95% of the tumor cells, the two other cases have a moderate expression in 30% and 50% of the cells.
Two cases show a low proliferation rate (MIB1 expression in less than 5% of the tumor cells), whereas the third case show a high proliferation rate (about 60% of the tumor cells positive for MIB1). This is the same case with high P53 accumulation.

3.2.5. FLC:

Seven of the 8 FLC cases (87.5%) show double positivity for HepPar-1 and CK7. The HepPar-1 positivity is typically strong, diffuse and with granular cytoplasmic pattern, whereas the CK7 positivity ranged from focal and moderate in one case to strong and diffuse in the remaining cases.
The eighth case shows positivity for HepPar-1 with negativity for CK7. All the cases (100%) show moderate to strong positive staining for FGG (Figure 7) either as individual strongly stained cells in 5 cases (62.5%) or as a diffuse

moderate cytoplasmic staining with strong positivity of individual cells in 3 cases (37.5%).

No SALL4 positivity observed in any of the FLC. The eight FLC show a positive membranous pattern of β-Catenin expression (100%) with focal cytoplasmic positivity in 4 cases. No nuclear positivity identified (0%). All the cases (100%) are immunoreactive for Wnt1.

No immunoreactivity with *BRAF* antibody identified.

4 cases (50%) show P53 expression (3 with moderate to strong nuclear accumulation in 1-2% of the cells and one with moderate nuclear accumulation in 5% of the cells).

5 cases (62.5%) have a mild proliferation activity in the MIB1 (<5% of the cells in 4 cases and in about 15% of the cells in one case).

3.3. ***Molecular analysis:***

3.3.1. HCC:

BRAF mutation status was determined in 50 of the 51 cases using the melting point analysis.

Two cases showed the typical mutational *BRAF* melting curve (the melting point of the sensor probe is about 56 °C for the WT sequence and 60 °C for the V600E). The cases were selected for confirmatory testing by Sanger sequencing which validated the results (Figure 8). The two mutations were represented by the most common substitution of valine by glutamic acid at position 600 (V600E).

Two additional cases showed an adjacent small peak in the melting curve suggesting of V600E, but in the sequencing analysis these were only representable in some of the alignments (or sometimes the high level of background noise did not allow the exact discrimination), also upon repeating the analysis from different blocks we got the same results. These findings might be interpreted by the fact that only a subset or sub clone of the tumor cells harbours the mutation.

3.3.2. CCC:

Genomic DNA from 31 of the 40 CCC was analysed for *BRAF* gene mutations. In the remaining 9 cases we had insufficient DNA to perform the analysis. Using

the Sanger sequencing method, we failed to detect specific gene mutations in exon 15, codon 600 in all the 31 cases.

3.3.3. Klatskin tumors:

Using Sanger sequencing, we did not detect *BRAF* mutations in any of the seven Klatskin tumors.

3.3.4. Combined HCC/CCC:

One of the three HCC/CCC cases shows a *BRAF* V600E mutation confirmed by both Sanger sequencing and melting point methods. Hereafter we isolated the DNA from the two different tumor components separately. Interestingly only the HCC tumor component shows to harbour the V600E mutation. The results were validated from a second tumor block.

3.3.5. FLC:

Of the eight FLC cases only one case show a melting point with a small doubtable adjacent peak. This was also not representable in all the sequencing alignments.

3.4. ***Long term outcomes (Table 8):***

3.4.1. HCC:

Follow-up information was available for 46 of the 51 patients. Seven additional patients did not present again after the operation discharge.

The length of the follow-up period ranged from 1-78 months (median, 26.7 months). Only one patient has documented death 32 months after the operation (case no. 34, Table 7). This patient has had HCV infection and liver transplantation and has died of liver failure after the activation of the HCV-infection with re-cirrhosis.

The Transcatheter Arterial Chemoembolization/ Transarterial Chemoembolization (TACE) technique was used in 7 (17.9%) cases (one cycle or more), which made it possible to assess pathologic response rate in these cases. Pathologic complete response (complete tumor necrosis) was not achieved in all cases.

Neoadjuvant therapy (NAT) was administered only in two cases (5.1%). The two cases have had multiple tumors or central large tumor with several satellite nodules. This was combined with TACE too.

Eleven patients (28.2%) developed local recurrence. The period to the recurrence ranged from 4-25 months (median, 11.6 months). The recurrent tumor was multifocal in three cases, was treated with Radiofrequency ablation (RFA) alone or combined with one or more cycles of TACE.

Six cases (15.4%) developed distant metastases (bone and soft tissues in 2 cases, peritoneal carcinosis in 2 cases, lymph nodes metastases in one case and disseminated metastases in one case too). These was treated with palliative chemotherapy alone or combined with the multi kinase inhibitor Sorafenib.

3.4.2. CCC:

Follow-up information was available for 34 of the 40 patients. Nine additional patients did not present again after the operation discharge.

The length of the follow-up period ranged from 1-81 months (median, 20.1 months). Four patients have documented death (period ranged from 8 to 62 months, median is 31.75 after the operation).

Neoadjuvant therapy (NAT) was administered in three cases (12%).

Eight patients (32%) developed local recurrence, of which 3 patients have had second recurrence metachronically. The period to the recurrence ranged from 4-43 months (median, 22.25 months), whereas the period to the second recurrence ranged from 14-50 months (median, 34 months). The recurrence was treated with chemotherapy or radiochemotherapy with or without Radiofrequency ablation (RFA).

Also eight cases (32%) developed distant metastases: bone and soft tissues in 2 cases, omentum and peritoneal carcinosis in 4 cases, lymph nodes metastases in 1 case and disseminated metastases (lymph nodes and lung) in one case too. These were treated with palliative chemotherapy.

3.4.3. Klatskin tumors:

Follow-up information was available for all the patients.

The length of the follow-up period ranged from 1-69 months (median, 27.1 months). Two patients have documented death at 7 and 9 months after the operation. These are patients with T4 and T3 tumors respectively (tumor infiltrates the main branch/ bilateral branches/ unilateral branches of portal vein or common hepatic artery) and the death was due to multi organ failure in the first patient and recurrent AML in the second one.

Neoadjuvant therapy (NAT) was not administered in any of the patients. Three patients (72.9%) developed local recurrence. The period to the recurrence ranged from 5-69 months (median, 30 months). One of these three patients developed peritoneal metastases concurrent to the recurrence (at 16 months) and a second recurrent tumor at 27 months and was treated with palliative chemotherapy (combination of Cisplatin and Gemcitabine).

3.4.4. Combined HCC/CCC:

Follow-up was available for the three patients. The length of the follow-up period ranged from 3-32 months (median, 17.3 months).

No patient was neoadjuvantly treated. All three patients developed local recurrence. The period to the recurrence ranged from 3-32 months (median, 17 months). The recurrent tumor was treated with Radiofrequency ablation (RFA) alone or combined with adjuvant chemotherapy. One of the three patients developed concurrent abdominal wall metastases.

3.4.5. FLC:

Follow-up information was available for 4 of the 7 FLC patients. The length of the follow-up period ranged from 2-90 months (median, 44.5 months).

One patient developed local recurrences at 15 und 44 months after the operation which were resected accordingly. At 90 months the patient is still disease free.

4. ***Discussion and Conclusion:***

4.1. *Histological and clinical findings:*

4.1.1. HCC

About 57% of the HCCs in this series are associated with cirrhosis. This emphasizes the role of chronic hepatic injury in the predisposition to HCC [139]. A viral association (HBV and HCV) is documented in about 20% of the cases (8% for HBV and 12% for HCV). This is much lower percentages as mentioned in the literature (50-55% with HBV, and 25-30% with HCV) [63, 3]. This could reflect the geographic variability in HBV and HCV prevalence and the effectivity of prophylactic procedures and vaccination programs. It could also represent a selection bias, because the selection of patients for surgical resection is based on clinical findings, laboratory data, imaging and staging systems, so that HCC patients with nonscirrhotic liver are most frequently selected as surgery candidates than those with cirrhotic background because of the lower morbidity rates.

About 88% of our patients are men (the sex incidence ratio is 7.5). This is a higher ratio than known incidence ratios in different parts of the world (varying from 1.3 to 3.6) [63]. The fact that sex ratios tend to be higher in high risk countries and patients less than 50 years of age [3] cannot really interpret this finding as Germany (Northern Europe) are among the geographic areas with low risk incidence.

The mean patient's age is nearly 65 years consistent with the mean age of developing HCC in developed countries [105] (usually after age 50).

4.1.2. ICC

Consistent with the published literature [51], the background liver showed a cirrhotic architecture only in 10% of our ICCs, reflecting the fact that ICC is less frequently associated with chronic hepatic injury than HCC, the cirrhosis was of non-biliary type in all the cases [127].

ICC was largely classified into conventional adenocarcinoma and other variants

and several studies reported these different histologic variants to be very rare [51, 143].
87.5% of the cases in our series show the conventional CC-morphology whereas the rest 12.5% of the cases are divided between mucinous, signet ring and clear cell carcinoma as rare morphologic variants.

The average age of patients diagnosed with intrahepatic CC is 70 and it is 73 for the extrahepatic CC [51]. But in our series the patients are nearly one decade younger, probably because of the different pathogenesis or small study sample.

4.1.3. FLC

In the study from El-Serag et al [155], the vast majority of FLCs has arisen in noncirrhotic livers and was not associated with other factors implicated in the development of HCC, such as HBV or HCV, alcohol consumption, or use of oral contraceptives. The mean age of patients was 39 years versus a mean age of 65 years in patients with HCC. FLC was also more common in women.
In our small series, none of the cases is associated with cirrhosis, no association with HBV or HCV documented, 4/7 patients are males, and the mean age at presentation is 31.4 years.

FLC is most often found as a solitary mass (80% to 90%) involving the left lobe of the liver [51]. The tumor is large at presentation, ranging in average size from 9 to 14 cm in greatest dimension [156].
The mean tumor size in our series is 13.6 cm.

In the study of Stipa et al [188], approximately one third of the resectable FLCs has lymphovascular invasion and 50% show lymph node metastases at the time of surgery.
In our series, three of four cases have N1 status, one case shows lymphatic invasion (L1) and one vascular invasion (V1).

The common presence of a central scar in FNH and FLC has previously led to the speculation that they share a common pathway of development. However,

only rarely have the two tumors been reported in association [173, 174]. In one of our FLCs, the tumor was associated with FNH.

4.2. *Immunohistochemical findings:*

4.2.1. Conventional immunostains:

Demonstrating the presence of bile canaliculi (using a polyclonal CEA antibody or CD10) in a tumor is considered diagnostic for an HCC, bile canaliculi are readily identified in most well- to moderately differentiated tumors but may be inconspicuous in high-grade HCCs (in about 50% of the cases), CD10 and p-CEA cross-react with canalicular biliary glycoproteins in 30% to 100% of HCCs [82], depending on the degree of differentiation of the tumor.
In our series about 70% (36/51) of the HCCs show typical canalicular expression of CD10, whereas none of the ICCs cross-react with the marker ($P<0.0001$). This highlights the importance of this marker in differentiating HCC from ICC, especially in small specimen liver biopsies.

HepPar-1 is expressed by HCC in a granular and cytoplasmic pattern and may be patchy within the tumor, with the sensitivity in recent series ranging from 73% [83] to 93% [84].
92% (46/51) of our cases are positive for HepPar-1 with negative cases more likely to show a poor differentiation. In contrary, HepPar-1 is positive only in two out of 40 ICC, i.e. ($P<0.0001$).

Glypican-3 is serological and histochemical marker of HCC. Yamauchi et al [82] reported the expression of Glypican-3 in 84% of HCCs whereas metastatic adenocarcinoma and cholangiocarcinoma stain rarely. In the series of Shirakawa et al [216], the Glypican-3 expression identified in about 78% of HCCs with different sensitivity to the different differentiation grades (60% of well differentiated, 90% of moderately differentiated and about 85% of poorly differentiated HCCs). Also this study confirmed that Glypican-3 expression is specific to HCC component of combined HCC/CCC.
The results in our review were a little higher (98% in HCC) which could be interpreted by the difference in the clone of the antibody.

Yamauchi et al [82] also recognized AFP as a highly specific but relatively insensitive marker, with positivity in only 17% to 68% of HCC cases [82]. In our review only 8% (4/51) of HCCs and none of the other cases (CCCs, FLCs, or CC component of HCC/CCC) are immunoreactive for AFP (P=0.027 in discriminating HCC from ICC).

The immunohistochemistry for cytokeratin 7 is known to be strongly positive in CCC with variable expression in HCC. Consistent with the literature, 95% (38/40) of our CCCs and about 19% of HCCs (10/51) express CK7 (P<0.0001). This emphasizes the utility of this marker in the differential diagnosis of primary liver malignancies, especially in the subset of CCCs which reveal a trabecular growth pattern mimicking HCC.

HCC genomes suffer extensive damage in the form of large-scale copy number alterations and viral integrations, which if left unchecked, would be expected to trigger TP53-mediated apoptosis and cell cycle arrests. Frequent mutations and deletions of TP53 appear to have disabled this important line of cellular defense.

Among the mutations involved in hepatocarcinogenesis identified in the cohort of Kan et al [92], TP53 has the highest prevalence (35.2%), consistent with earlier HCC studies [57, 217]. These tumors are more likely to be poorly differentiated and have poor survival.

Overexpression of mutated p53 can be detected by immunohistochemistry in up to 37% of HCCs [77]. In our review it was detected in about 26% of the cases and was significantly correlated with higher tumor grade (P=0.0019).

P53 mutations are also involved in Cholangiocarcinogenesis. 85% of our ICC cases show nuclear accumulation of P53 either as focal, moderate, or strong accumulation. P53 expression was also statistically correlated with higher tumor grade (P=0.0052).

The molecular basis for FLC remains largely unknown. Only a limited number of individual oncogenes and signaling pathways have been studied in FLC, but the results indicate that the uniqueness of FLC extends to the molecular level.

Several studies showed that FLC does not share the genetic abnormalities that have been demonstrated in conventional HCC, such as p53 mutations, survivin

overexpression [176, 177], β-Catenin mutation, etc [57, 59, 156].
Similar to these findings we observed only focal P53 accumulation (in <5% of the tumor cells) in 4 FLCs in our review.

Ki-67 is a nuclear antigen expressed in the G1, S and G2 phases. Overexpression of Ki-67 suggests cell cycle control disturbances and increasing proliferation.
Proliferative activity measured by positivity for nuclear Ki-67 antigen (clone MIB1) increases with decreasing degrees of HCC differentiation but cannot distinguish between low-grade HCC and benign nodules [218]. Ki-67 is also a potentially valuable prognostic factor in patients with HCC. HCV-related HCC does have lower proliferative activity and a better prognosis.
Regarding Ki-67 expression in CCC, the study of Shrestha et al [219] noticed increased expression in the extrahepatic CC than in other bile duct carcinomas. This expression was also higher in poorly differentiated tumors and lymph node metastasis group. Ki-67 was found to be a good prognostic indicator whereas there was no association of p53 and MIB1 expression.

Our study did not notice any significant association between MIB1 and any of the clinicopathological parameters in both HCC and CCC cases.

4.2.2. Aberrant Wnt/β-Catenin pathway in hepatic malignancies:

HCC is one of the cancers with a high rate of dysregulation in the Wnt/β-Catenin pathway, as 40%-70% [220-223] of HCC patients have tumours with high levels of β-Catenin accumulation.
Maher et al [202] reported that β-Catenin phosphorylated at Ser45 is predominantly located in the nucleus, whereas β-Catenin phosphorylated at Ser33/Ser37/Thr41 is mostly localized to the cytoplasm. This suggests that phosphorylation at Ser45 and at Ser33/Ser37/Thr41 is not necessarily coupled. It may also imply that phosphorylation at Ser45 by CKIα serves another function, yet to be delineated, other than priming β-Catenin for further phosphorylation by GSK3β.
Nuclear accumulation of β-Catenin is strongly associated with β-Catenin mutations [221]. A majority of β-Catenin mutations in HCC are missense mutations occurring at exon 3 (at the sites of GSK3β phosphorylation -Ser45,

Ser33, Ser37 and Thr41-, are deletions in β-Catenin, or occur at other sites). This region is responsible for phosphorylation and ubiquitination of β-Catenin, and therefore, mutation in this region results in stable β-Catenin that consequently accumulates in the nucleus.

Kan et al [92] documented β-Catenin (CTNNB1) mutations (exon 3) in 15.9% of the HCCs in their series.

The study detected also a small deletion in exon 3 of CTNNB1 that removes the GSK3β phosphorylation sites (S33 and S37). Moreover they showed that negative regulators of CTNNB1 including AXIN1 (4.5%), AXIN2 (2.3%) and APC (2.3%) could be also mutated, implicating the canonical Wnt pathway as a major driver of hepatocarcinogenesis.

Mao et al [224] associated nuclear β-Catenin accumulation to β-Catenin mutation, non-invasive form of tumour and good prognosis. HCC tumours with mutant nuclear β-Catenin resulted in a better 5-year survival than HCC tumours with wild-type nuclear β-Catenin accumulation. This is suggestive of the statement that wild-type β-Catenin accumulation and mutant β-Catenin accumulation are not equivalent.

However, several studies have correlated nuclear β-Catenin accumulation to tumour progression and poor prognosis [223, 225, 226].

Kondo et al [226] reported that β-Catenin accumulation and β-Catenin mutation do not occur early in hepatocarcinogenesis, but could be associated with malignant progression of HCC. Similar to these findings, Inagawa et al [225] observed poor prognosis in HCC patients with nuclear β-Catenin accumulation in grade 3 HCC tumours and not in grade 1 or grade 2 HCC tumours. Furthermore, nuclear β-Catenin accumulation in HCC has also been correlated to tumour cell proliferation (Ki67 expression), suggesting that β-Catenin promotes tumour progression [223]. The discrepancy in β-Catenin accumulation and HCC prognosis could be due to the type of β-Catenin mutations. Other reasons for the discrepancy may include tumour histology and the size of the tumour.

Additionally, the presence of β-Catenin mutations demonstrates different phenotypical features in HCC. Cieply et al [227] reported that HCC tumours harbouring a missense mutation at exon 3 exhibit a more aggressive phenotype and may develop HCC without cirrhosis compared to HCC with non-mutated β-

Catenin. Thus, β-Catenin mutations may serve as an independent risk factor for the development of HCC in the absence of cirrhosis.
Greater tumour size has also been reported in HCC tumours with β-Catenin mutations as compared to those without mutation in β-Catenin [228].
Some studies have correlated cytoplasmic β-Catenin (non-nuclear β-Catenin) with poor cellular differentiation, large tumour size (> 5 cm in diameter) and short disease-free survival [221]. For reasons not yet elucidated, HCV-associated HCC has a greater frequency of β-Catenin mutations than the HBV-associated type [222].
Several studies on transgenic animal models have shown that overexpression of mutant or stable forms of β-Catenin on its own is not sufficient to induce tumours in liver [229-231]. However, deletion of APC in mice results in hepatomegaly, hepatocyte hyperplasia and rapid mortality [232]. Thus, β-Catenin mutations or accumulation may cooperate with other genes or signalling pathways to result in hepatocarcinogenesis.

Wnt family is composed of nineteen secreted glycoproteins [233]. They bind to the extracellular domain of FZDs and activate the Wnt/β-Catenin pathway [234]. Ten different FZD genes have been identified in mammals and all of them encode seven transmembrane receptors [235]. Wnt1 is upregulated in HCC tissues compared to adjacent non-tumour tissues and its expression has been associated with tumour recurrence [236]. Furthermore, three other Wnt genes (Wnt3, Wnt4 and Wnt5A), and three FZD genes (FZD3, FZD6 and FZD7) are also upregulated in HCC tissues and preneoplastic peritumoural tissues as compared with normal liver tissues, suggesting that their overexpression may be an early event in hepatocarcinogenesis. However, only the overexpression of FZD7 has been associated with nuclear and/or cytoplasmic accumulation of β-Catenin in HCC [237-239].

In our series of HCCs, about 20% of the cases show nuclear beta-Catenin positivity, whereas diffuse and strong granular cytoplasmic Wnt1 positivity is evident in about 94% of the cases.
There was no correlation between β-Catenin expression and any of the clinicopathological features, whereas this expression was statistically correlated

with the Wnt1 expression (P=0.034).
Wnt1 expression was conversely correlated with P53 positivity as about 72% of the positive cases show no P53 accumulation (P=0.012).
This finding suggests that these two pathways could be mutually exclusive in contributing to HCC tumorigenesis.

Several reports indicate that Wnt/β-Catenin pathway contributes also to cholangiocarcinogenesis (partially through the role of β-Catenin in E-cadherin mediated cell-to-cell adhesion). Sugimachi et al [240] observed reduced membranous expression of β-Catenin in 82% of their examined ICCs and indicated that this reduction of expression is associated with non-papillary ICCs which have a more malignant behaviour. They suggested this reduction leads to loss of a cell-to-cell adhesion, an event which may contribute to the invasive tendency of ICC.
The study also observed nuclear accumulation of β-Catenin in 15% of the ICCs, but mutations in β-Catenin exon 3 do not appear to be responsible for this nuclear translocation of β-Catenin (all the cases examined showed the WT sequence) and therefore indicated that the manner in which β-Catenin is translocated into the nucleus in cholangiocarcinoma cells is not clear, and events other than mutations of β-Catenin may be responsible.
On the other hand, Zahng et al [241] investigated the roles and mechanisms of MicroRNA-26a (miR-26a) in human cholangiocarcinoma as miRNAs have been recently implicated in the development and progression of human cancers.
According to their results, human cholangiocarcinoma tissues and cell lines had increased levels of miR-26a compared with the noncancerous biliary epithelial cells. Overexpression of miR-26a increased proliferation of cholangiocarcinoma cells and colony formation in vitro, whereas miR-26 depletion reduced these parameters. Furthermore, GSK-3β messenger RNA was identified as a direct target of miR-26a by computational analysis and experimental assays. Thus miR-26a promotes cholangiocarcinoma growth by inhibition of GSK-3β and subsequent activation of β-Catenin and these signaling molecules might be targets for prevention or treatment of cholangiocarcinoma.

We evaluated the cellular localization of β-Catenin in CCC from two different

points of view; membranous and nuclear expression pattern, since these patterns reflect the dual function of β-Catenin which involves both cadherin mediated cell-to-cell adhesion and Wnt signaling pathways. Unlike the observations of Sugimachi and the similar studies [242], we did not observe any reduced membranous expression of β-Catenin in any ICC or Klatskin case whereas a reduced membranous expression was observed in one of the combined HCC/CCC cases.

We immunohistochemically detected strong nuclear accumulation of β-Catenin in 7.5% of the ICCs we studied herein and this nuclear expression was significantly correlated with the presence of vascular invasion (P=0.048) and with tumor localization in the left hepatic lobe (P=0.018). No correlation between this expression and the other clinicopathological features noted.

Also 85% of the ICCs were reactive for Wnt1. Wnt1 expression in ICC was not significantly correlated with any of the clinicopathological parameters.

As already mentioned FLC does not share the genetic abnormalities that have been demonstrated in conventional HCC including β-Catenin mutation.

While no β-Catenin mutations are found in FLC in the study from Terris et al [243] and there was no nuclear accumulation of β-Catenin by immunostain, Cieply et al indicated there is some evidence that the Wnt signaling pathway may still be active in FLC [227].

Similar to these findings nuclear β-Catenin accumulation has not been observed in any of our 8 FLCs, whereas Wnt1 stain (as a probable indicator for Wnt signaling pathway activation) was strongly positive in all the FLCs.

4.2.3. SALL4 expression:

Sal-like protein 4 (SALL4) is a member of a family of zinc finger transcription factors. It is a regulator of embryogenesis, organogenesis, pluripotency, can elicit reprogramming of somatic cells, and is a marker of stem cells. It's expression is noted in normal murine hepatoblasts, normal human hepatic stem cells, hepatoblasts and biliary tree stem cells, but not in mature parenchymal cells of liver or biliary tree (silenced in the adult liver) [244].

Experimental manipulation of SALL4's expression results in changes in proliferation versus differentiation in human HCC cell lines in vitro and in vivo in

immune-compromised hosts. Virus-mediated gene transfer of SALL4 was used for gain- and loss-of-function analyses in the cell lines. Significant growth inhibition in vitro and in vivo, accompanied by an increase in differentiation occurred with down-regulation of SALL4 [244], partly through released suppression of the phosphatase and tensin homologue protein (PTEN) gene [245].

The absence of SALL4 expression in the healthy adult liver enhances the potential of SALL4 as a treatment target in HCC [245].

About 8% (4/51) of the HCC cases in our series show positivity for SALL4. This was typically granular to punctuate/clumped nuclear positivity. All the cases have embryonic stem-cell features and show probably an aggressive behaviour (this is a small series of cases to be correlated with patients' survival, especially that two patients did not present after the operation discharge, but one of the other two patients developed lymph nodes metastases six months after the operation and the another developed multiple tumor nodules which necessitated neoadjuvant and additional surgical interventions).

Statistically there was a significant correlation between SALL4 expression and some of the clinicopathologic characteristics: higher histologic grade (P=0.040), higher pT stage (P=0.035) and vascular invasion (P=0.022).

Moreover, SALL4 expression significantly correlates with higher serum AFP levels (P=0.026) and with the loss of immunohistochemical expression of Wnt1 (P<0.0001).

Also focal to diffuse finely granular to punctuate positivity in 7.5% of the ICC (3/40) was observed, of which one case developed local recurrence, the second multiple local recurrences, whereas no follow up data was available for the third case.

This immunohistochemical expression was statistically correlated with the presence of multiple tumor nodules (P=0.0035), with the presence of vascular invasion (P=0.048) and with higher recurrence rate (P=0.035).

Interestingly 2 of the three HCC/CCC cases showed clumped nuclear positivity mainly in the HCC tumor component. The two cases developed local recurrences which were accompanied in one of them by metastases in the

abdominal wall.
None of the Klatskin tumors or of the FLCs was immunoreactive for SALL4.

No SALL4 expression was observed in the normal liver parenchyma in any of the cases in this series.

Unlike some previous studies that reported lack of SALL4 expression in HCC and gastric cancer [246-248], this is one of the first reports documenting for the SALL4 expression in liver cancer.
This could be potentially attributed to differences in clone and source of antibody used.

Gonzalez-Roibon et al [249] reported the expression of SALL4 in 7% of their HCC series using the 25% cutoff and in 27% of the cases using the 5% cutoff. They also did not identify any SALL4 expression in the nonneoplastic liver samples. The study did not find any correlation between SALL4 extent of expression and any of the clinicopathologic characteristics (histologic grade, tumor focality, vascular invasion, tumor size, and pT stage).

In the series from Oikawa et al [244] SALL4 was strongly expressed in surgical specimens of human HCCs (17/20) and CCCs (4/5). The study also found that SALL4 is expressed in combined hepatocellular and cholangiocarcinoma and in a transplantable human tumor line derived from a FLC. SALL4 was not detected in chronic hepatitis but faintly detected in bile ductules and in hepatocytes at the interface of parenchymal and stromal cells in liver cirrhosis.
Accordingly the study suggested that SALL4 expression indicates selection for stem cells as a minor cell population in normal tissue and cirrhotic tissues and as a dominant cell population in liver cancers.
The performed bioinformatics analyses indicated that elevated expression of SALL4 in tumors is associated with poor survival of HCC patients [244].

4.2.4. FGG expression:

Eosinophilic hyaline globules and the so called cytoplasmic pale bodies or ground-glass inclusions are present in approximately half of cases in FLC.

These inclusions contain fibrinogen, which is highlighted by immunoperoxidase staining [250].
We observed immunohistochemical reactivity for Fibrinogen gamma chain (FGG) in all the FLC cases with a unique pattern of staining either as individual strongly stained cells in a non-stained tumor background or as diffuse moderate cytoplasmic staining with individual cells more strongly stained.

Fibrinogen is an important protein for coagulation. It is a hexameric protein made of two copies of three peptide chains: Aα, Bβ and γ. These chains are expressed and secreted as an assembled hexamer (AαBβγ)2 from hepatocytes. The three fibrinogen chains are encoded by three genes: FGA for Aα, FGB for Bβ and FGG for γ (with the molecular location on chromosome 4: base pairs 155,525,285 to 155,533,901). These genes (FGB-FGA-FGG) are expressed almost exclusively in hepatocytes where their output is coordinated to ensure a sufficient mRNA pool for each chain and maintain an abundant plasma fibrinogen protein level [251].
Studies on the control of fibrinogen gene expression have been ongoing for over 30 years.
During an acute phase inflammatory response, stimuli of expression include glucocorticoids, IL-1β and IL6.
Crabtree and Kant [252, 253] demonstrated increased liver fibrinogen mRNA dependent on glucocorticoids confirming the influence of steroids on fibrinogen expression that was described previously [254].
A second important finding was the stimulating effect of IL-6 (previously known as hepatocyte stimulatory factor, HSF) on hepatocyte fibrinogen expression. IL-6 stimulates a coordinated increase in fibrinogen mRNA and protein expression (the majority of regulation felt at the transcriptional level), which leads to STAT3 activation [255, 256].
In addition, miRNA can influence expression post-transcriptionally [219].
It is yet not clear if the FGG expression in FLC is the direct impact of mutations in the fibrinogen genes or as a result of disorders in other regulatory mechanisms mentioned.
All our FLC cases show positivity for FGG, whereas only 5/51 HCCs express the marker. This finding supports the utility of FGG in FLC diagnosis, especially

in the cases with less prominent background fibrosis or in discriminating FLC from HCC with desmoplasia –the so-called scirrhous type- (P<0.0001 in discriminating FLC from HCC).

4.3. *Molecular findings:*

BRAF mutations:

Only few data have been previously reported about any possible role of MAPK pathway and *BRAF* mutation rates among patients with primary hepatic malignancies.

Colombino et al [257] elucidated unexpected *BRAF* and *PIK3CA* mutations in HCC patients originating from South Italy. Overall, they detected oncogenic mutations in 23% for *BRAF* gene (V600E), 28% for *PIK3CA* gene, and 2% for K-*RAS* gene. Using statistical analysis, *BRAF* mutations were significantly correlated with the presence of either multiple HCC nodules (P=0.021) or higher proliferation rates (P=0.034).

These findings clearly indicated that mutational activation of both *BRAF* and *PIK3CA* genes does contribute to hepatocellular tumorigenesis at somatic level in Southern Italian population.

On the other hand, Tannapfel et al [258] identified activating *BRAF* missense mutations in 22% of their ICCs and in one case of tumour surrounding liver, whereas these mutations were not detected in the HCC tumors in their series. The study failed to observe a correlation between *BRAF* mutations and histopathological factors or prognosis of patients.

Recent studies into the molecular background of FLC have revealed overexpression of genes in the *RAS*, MAPK, *PIK3*, and xenobiotic degradation pathways [175].

We documented *BRAF* mutation V600E in 8% (4/50) of our HCCs including 4% (2/50) as “low level mutations” with small reproducible mutational peak which was not representable in all specimens obtained from different tumor blocks, in 1/8 FLC (also as low level), and in 1/3 compound HCC/CCC interestingly only in

the HCC tumor component. We failed to detect any *BRAF* V600E mutation in the other CCC cases analyzed (31 ICC and 7 Klatskin tumors). Using the commercially available antibody we could not identify any immunohistochemical positivity correspondingly.

Using a statistical analysis, *BRAF* mutations were significantly correlated with some clinicopathologic features of the HCC such as higher tumor grade (P=0.040) and tumor localization when it involves the both hepatic lobes (P=0.40).
An association was also noted with positive serology for HCV and HBV (P=0.038).
The mutational status did not correlate with the immunohistochemical expression from other markers.
Several new studies also found *BRAF*-mutations to be very rare in CCC [259, 260].

Table 9 summarizes all statistically significant correlations.

4.4. *Summary:*

We report the immunohistochemical and molecular findings from a series of primary hepatic carcinomas, aiming for novel markers which may add to their diagnosis and gain more understanding of genetically altered genes and pathways implicated in their geneses.

Regarding the standard immunostains applied, our findings are consistent with published literature and do strongly support the use of a panel of immune markers in the discrimination of primary liver malignancies.

We also focused on the biological significance of an activated β-Catenin pathway (identified by Wnt1 immunoreactivity and the nuclear translocated β-Catenin) in hepatocarcinogenesis that can open the door to evaluate existing inhibitors of this pathway for future therapeutic management.
It remains to be determined which genomic lesions identified by aberrant Wnt1 expression in these tumors are translated into nuclear β-Catenin accumulation.

The study documented the expression of SALL4 as a stem cell marker in this series. Our findings suggest that SALL4 may play a role in recognizing the primary liver cancers, especially the poorly differentiated “primitive” cases. In addition SALL4 is one of the potential treatment targets in liver malignancies, especially that its expression is absent in the healthy adult liver.

A unique type of expression of FGG immunostain was also observed in fibrolamellar carcinomas, the finding that supports the utility of FGG in FLC diagnosis, especially in the cases with less typical morphology or in discriminating FLC from scirrhous HCC.

This review also identified *BRAF*-V600E gene mutations in HCC, FLC and HCC component of HCC/CCC but not in other CCC cases. This provides a potential path toward therapeutic intervention of the disease.
We failed to identify a corresponding immunoreactivity with the commercially available *BRAF* antibody in the mutant cases. The conclusion is that the antibody is not a useful surrogate to detect *BRAF*-V600E mutations in primary liver malignancies.

From a statistical point of view, this is a small-sample study, but we hope that we will contribute to a meta-analysis combining results of similar across studies of those immunohistochemical and molecular findings in an adequately sized study.

Legends:

Figure 1.
Global variation in liver cancer incidence rates. From Parkin DM, Bray F, Ferlay J et al. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55: 74–108.

Data are estimates for the year 2002 from the GLOBOCAN database of the International Agency for Research on Cancer. Classification of incidence was based on data in males (a>20 per 100,000, b 11–20 per 100,000, c 5–10 per 100,000, d<5 per 100,000).

Figure 2.
Different Cytoplasmic Deposits and Inclusions in HCC: (a.) Fat droplets within an HCC (HE Stain, Magnification 20X), (b.) Mallory hyaline, representing clumps of intermediate filaments and found in about 20% of cases without relation to the underlying liver disease (HE Stain, Magnification 40X), (c.) Globular proteinaceous eosinophilic inclusions are also seen in 20% of cases and could represent A1AT (HE Stain, Magnification 40X), (d.) Glycogen accumulation in the cytoplasm of HCC cells responsible for the so called “clear cell variant” (HE Stain, Magnification 40X).

Figure 3.
Wnt/β-catenin signalling in the absence and presence of Wnt stimulus. A: Wnt/β-catenin signalling is regulated by several antagonists to prevent the formation of frizzled (FZD)-Wnt-low-density lipoprotein receptor-related protein 5/6 (LRP5/6) complex. Secreted frizzled-related protein (sFRP) and Wnt inhibitory factor (WIF) bind directly to Wnt, whereas dickkopfs (DKKs) bind to LRP5/6. Furthermore, human homologue of Dapper (HDPR1) and Prickle-1 inhibit the action of dishevelled (Dvl). In the absence of Wnt stimulus, β-catenin is first primed for phosphorylation by casein kinase Iα (CKIα) followed by phosphorylation by glycogen synthase kinase 3β (GSK3β) at three residues. The phosphorylated β-catenin is targeted for ubiquitination by β-transducin repeat-containing protein (β-TrCP) and is subsequently degraded by the proteasome. In the nucleus, T-cell factor (TCF)/lymphoid enhancer factor (LEF) represses transcription of the Wnt/β-catenin pathway target genes by interacting with co-repressor Groucho; B: Wnt binds to and activates FZD and LRP5/6 receptors. Dvl is recruited to the plasma membrane and binds to FZD. This results in the recruitment of Axin and GSK3β to LRP5/6. LRP5/6 is then phosphorylated by CKIα and GSK3β, resulting in an inactivation of the destruction complex and leading to β-catenin accumulation in the cytoplasm. β-catenin then subsequently translocates to the nucleus where it binds with TCF/LEF and other co-activators e.g. CREB binding protein (CBP) to mediate transcription of genes and microRNAs responsible for proliferation and growth. APC: Adenomatous polyposis coli; NLK: Nemo-like kinase; p: Phosphorylated; Ub: Ubiquitinated.

Figure 4.
Simplified illustration of the main signal ways. MAPK pathway plays a central role.

Figure 5.
The histological patterns of HCC: (a.) Pseudoglandular pattern (HE Stain, Magnification 10X), (b.) the trabekulär pattern (HE Stain, Magnification 10X), (c.) the solid pattern is seen in 5% to 15% of HCCs (HE Stain, Magnification 10X), (d.) the spindle cell variant may be more common in tumors subjected to chemo-embolization or preoperative chemotherapy (HE Stain, Magnification 20X).

Figure 6.
SALL4-Expression in hepatic cancer: (a.) The positive HCC cases show typically embryonic features (HE Stain, Magnification 10X), (b.) SALL4 positivity in an HCC, note the typical clumped nuclear expression (SALL4 Stain, Magnification 20X), (c.) nuclear SALL4 expression in CCC (SALL4 Stain, Magnification 20X), (d.) combined HCC/CCC, note the punctuate dot-like positivity (SALL4 Stain, Magnification 40X).

Figure 7.
Fibrolamellar carcinoma, (FLC): (a.) the tumor is characterized by thick fibrous bands in its Stroma (HE Stain, Magnification 10X), (b.) the positivity of FGG stain in FLC (FGG Stain, Magnification 20X), the cases show typically double positivity for HepPar1 (c.) (HepPar1 Stain, Magnification 10X) and CK7 (d.) (CK7 Stain, Magnification 10X).

Figure 8.
Examples of the results of BRAF somatic mutations. Top: a melting point from a positive HCC sample (case no. 34, Table 8): 1; WT curve, 2; positive control, 3; negative control, 4; patient sample. Bottom: Electropherogram shows the nucleotide sequences of the genomic DNA from the same sample; arrow indicates the mutation position within the sequence.

Table 1.
Studies reporting the age-standardized incidence of hepatocellular carcinoma (HCC) in the general population

Table 2.
Factors Implicated in the Pathogenesis of Hepatocellular Carcinoma.

Table 3.
Histologic Grading of Hepatocellular Carcinoma.

Table 4.
Hepatocellular Carcinoma--Cytoplasmic Deposits and Inclusions.

Table 5.
pTNM Staging of Primary Hepatic Epithelial Malignancies.

Table 6. (A and B)
Clinicopathologic (A), immunohistochemical and molecular (B) results in overview.

Table 7.
Clinicopathologic features of the hepatic tumors.

Table 8.
Follow-up results.

Table 9.
Significant statistical correlations.

Figure 1: Global variation in liver cancer incidence rates [5].

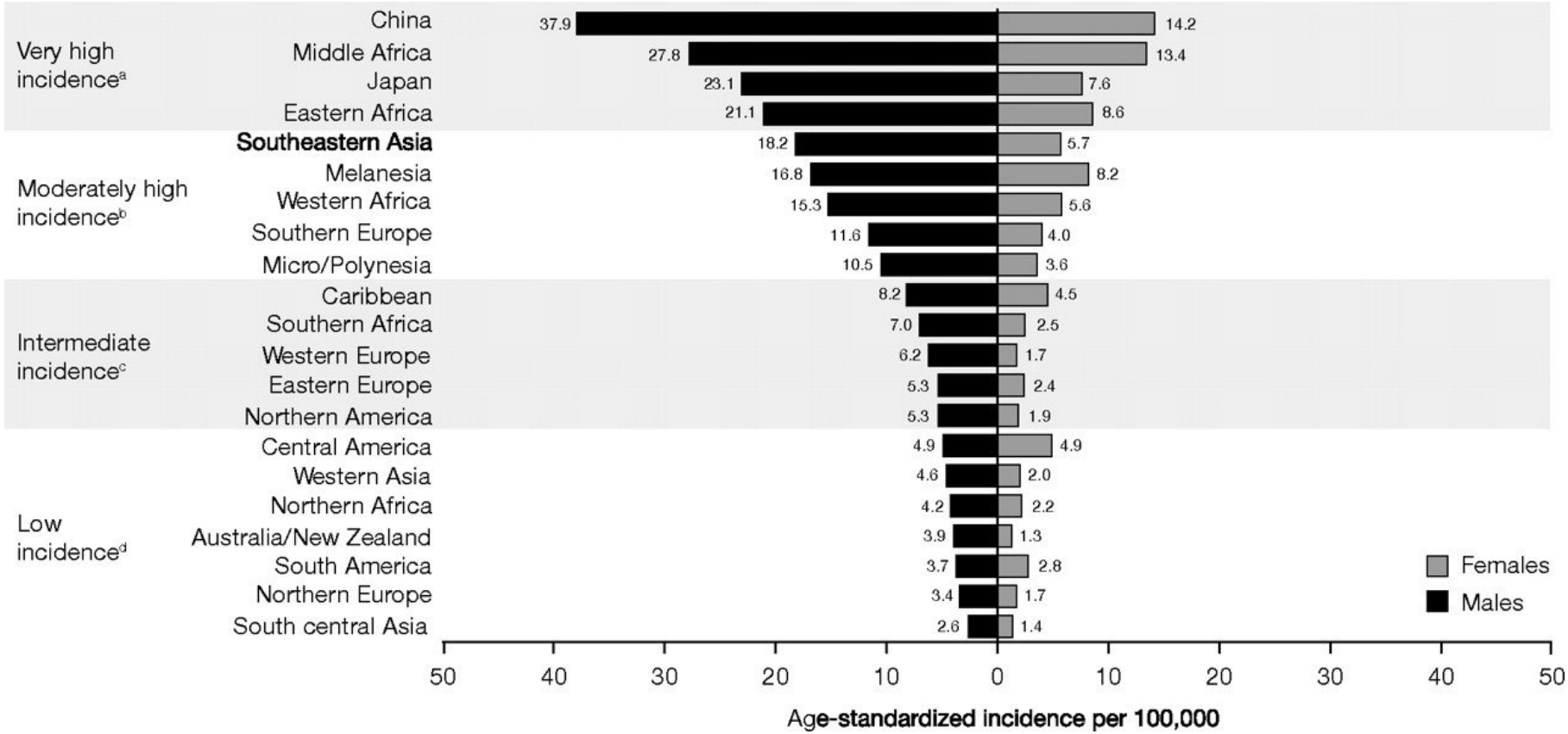


Figure 2

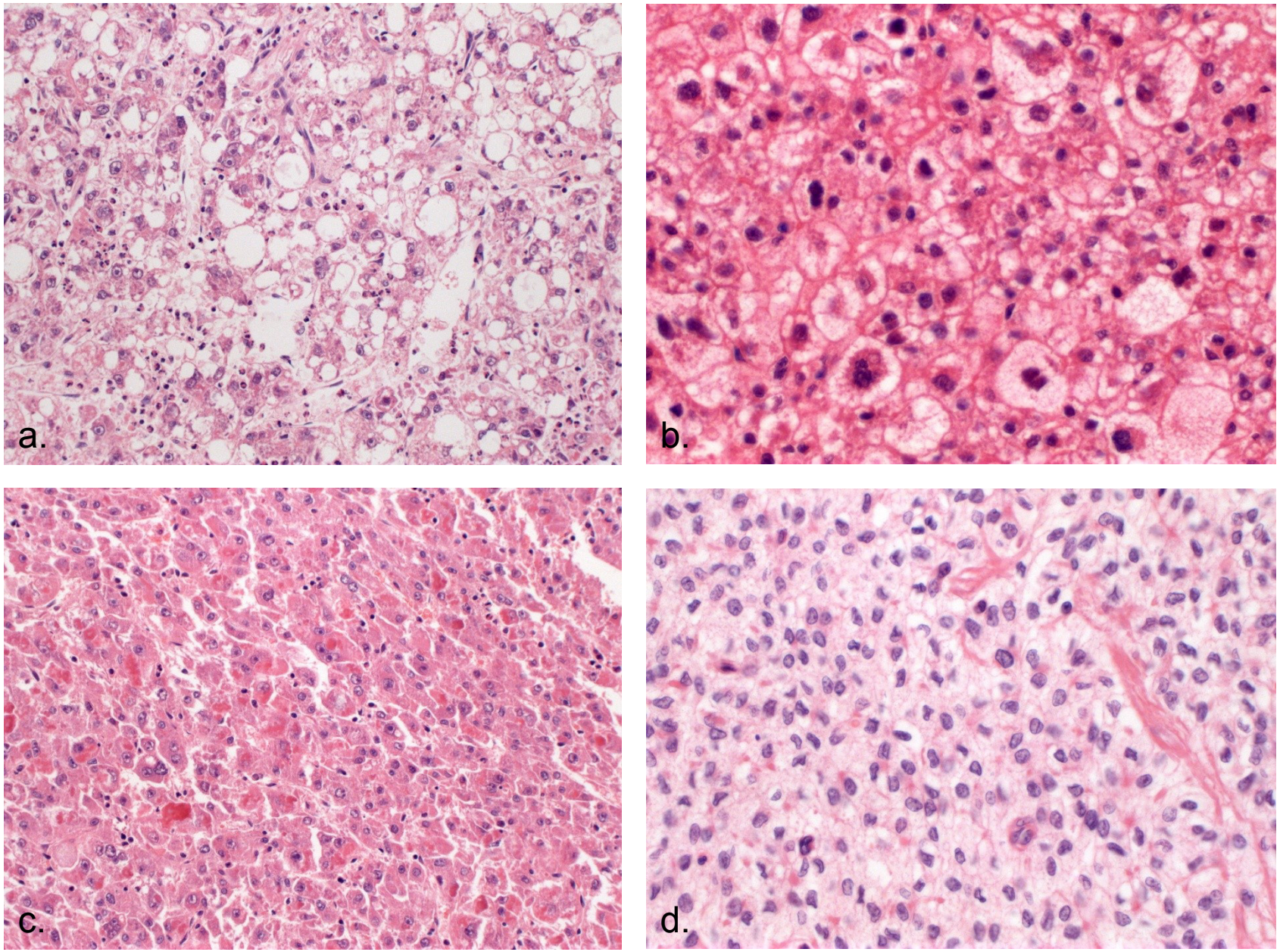


Figure 3

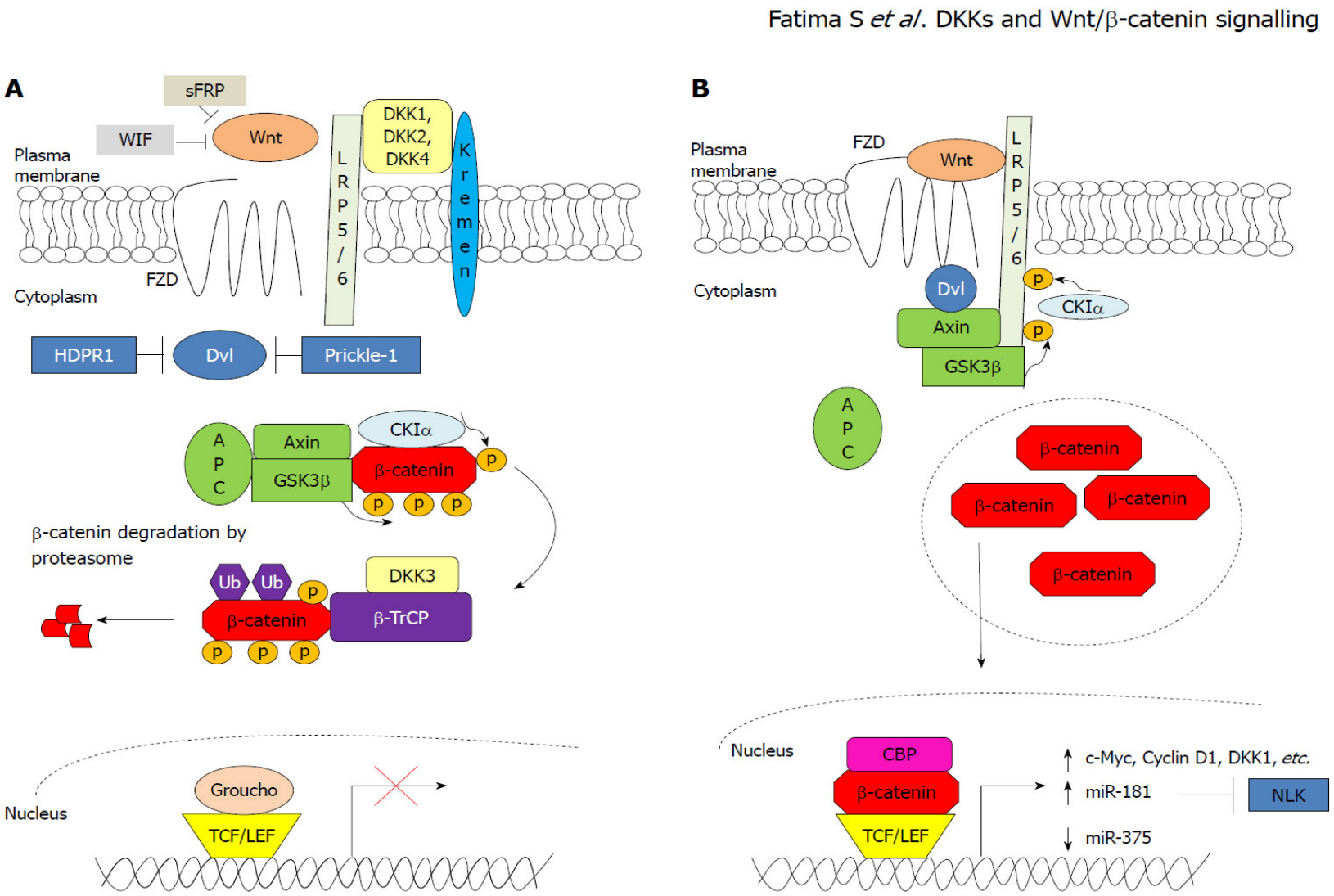
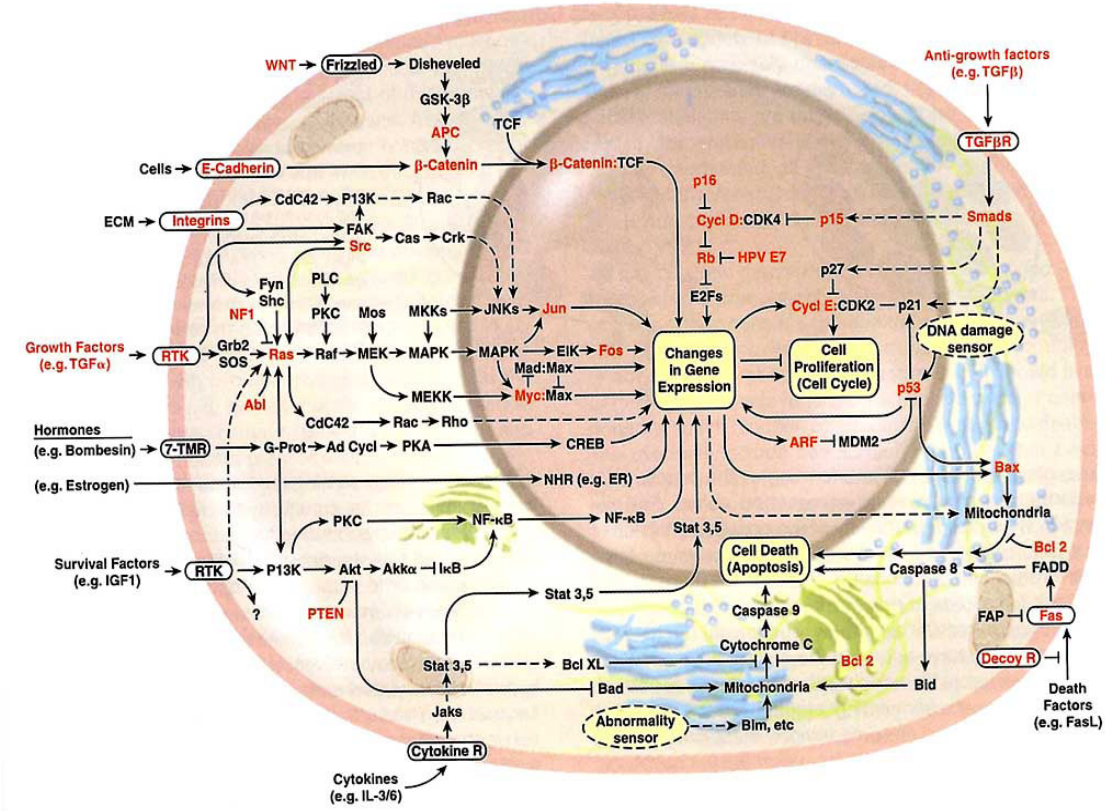
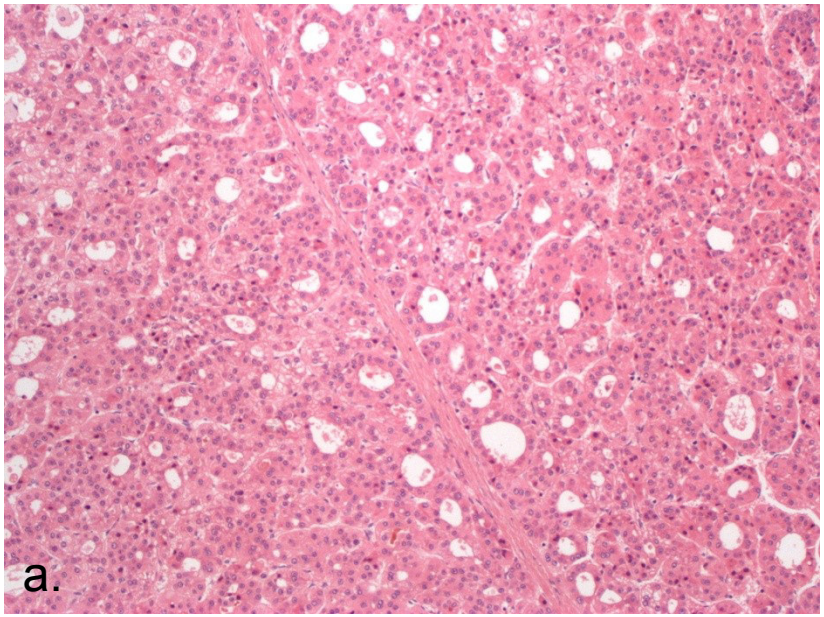

Fatima S *et al*. DKKs and Wnt/β-catenin signalling
A
sFRP
WIF
Wnt
DKK1, DKK2, DKK4
Kremen
LRP5/6
Plasma membrane
FZD
Cytoplasm
HDPR1
Dvl
Prickle-1
APC
Axin
GSK3β
CKIα
β-catenin
p
β-catenin degradation by proteasome
Ub
DKK3
β-TrCP
Nucleus
Groucho
TCF/LEF
B
Plasma membrane
FZD
Wnt
LRP5/6
Cytoplasm
Dvl
CKIα
Axin
GSK3β
APC
β-catenin
Nucleus
CBP
c-Myc, Cyclin D1, DKK1, *etc.*
miR-181
NLK
miR-375
TCF/LEF

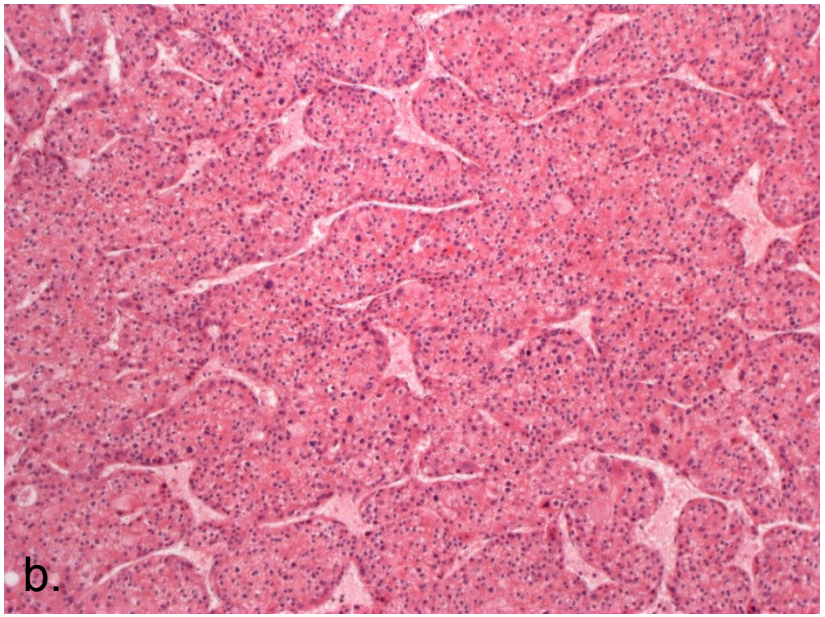
Figure 4

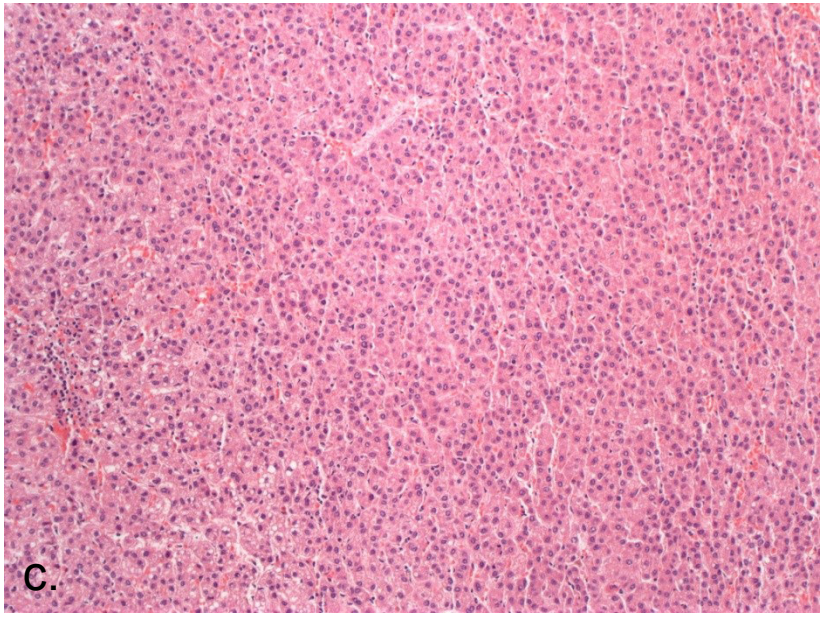


Mccubrey JA *et al.* Simplified illustration of the main signal ways. MAPK pathway plays a central role.

Figure 5







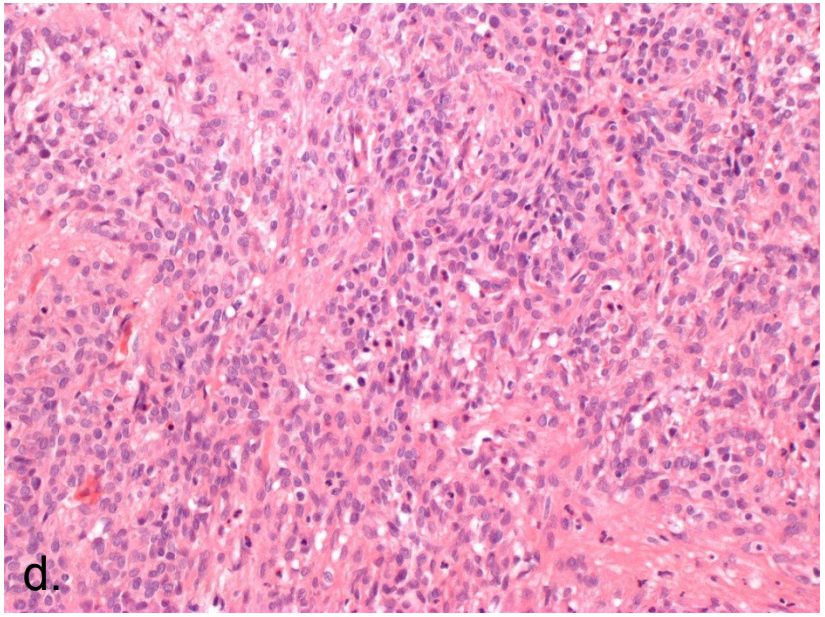


Figure 6

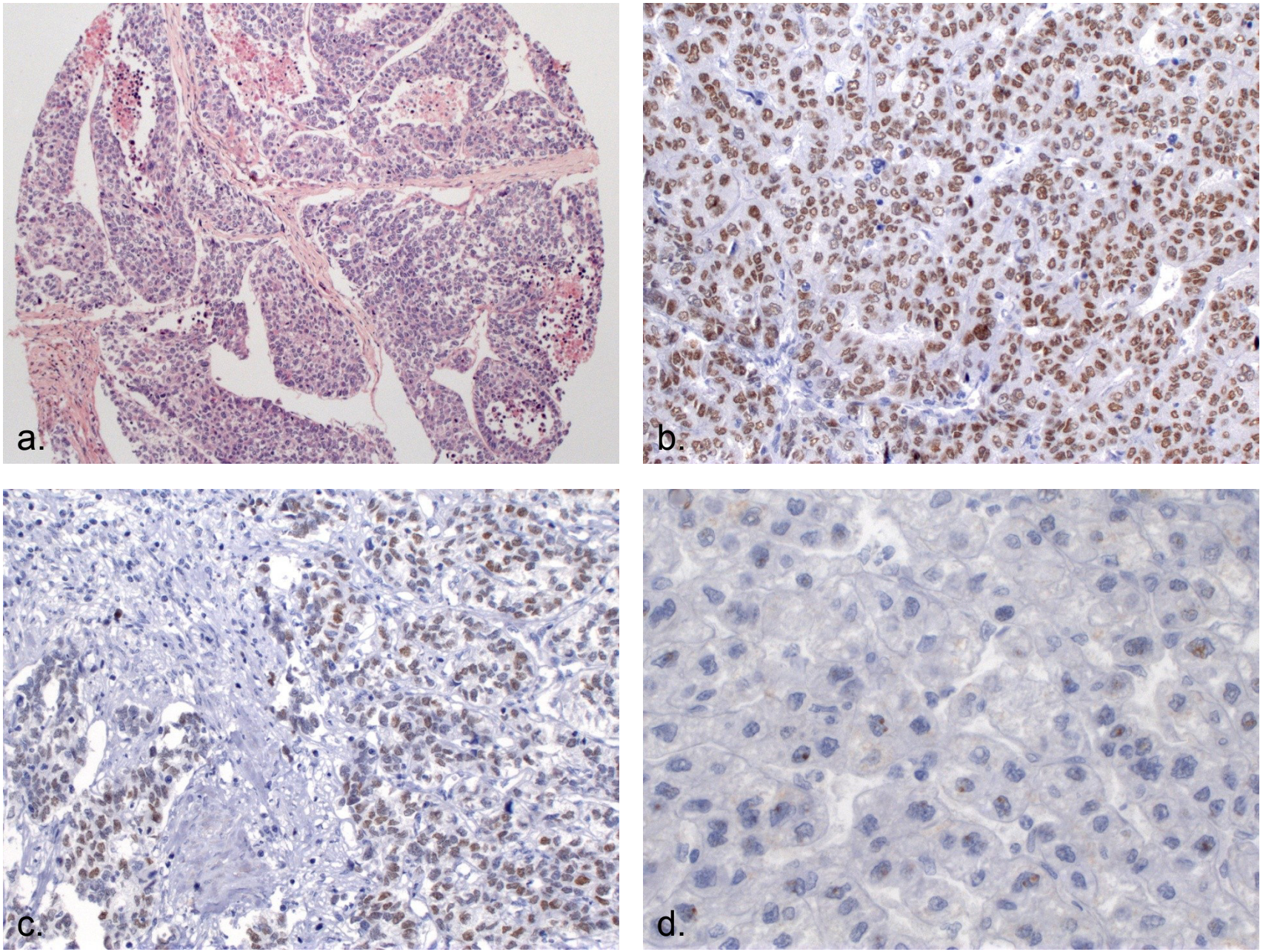
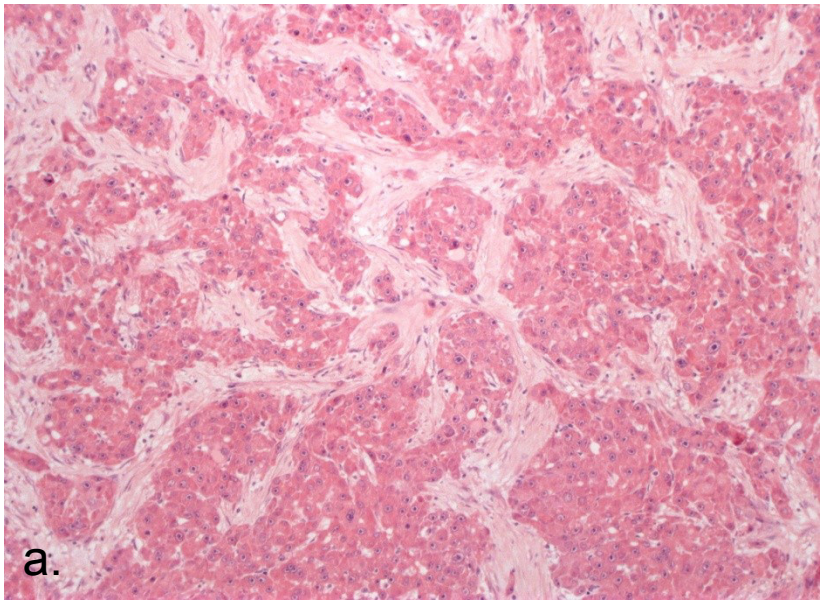
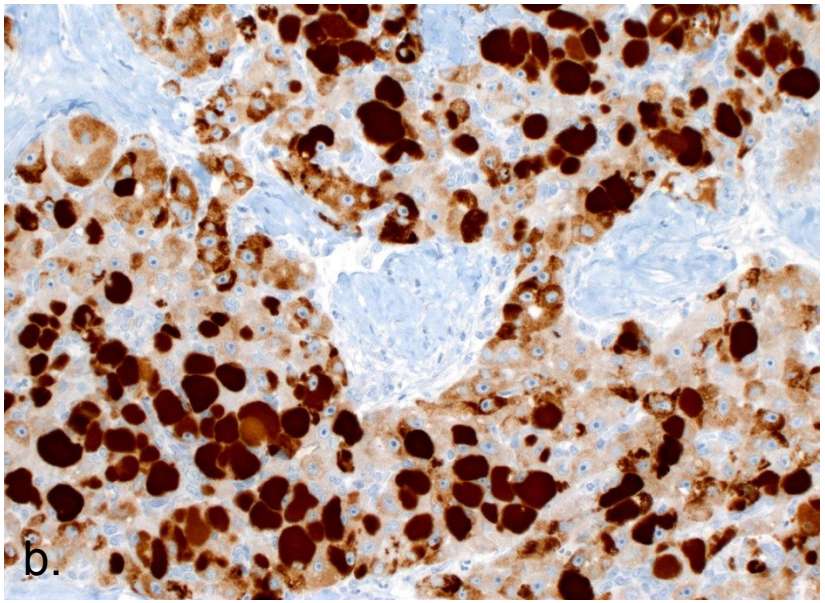
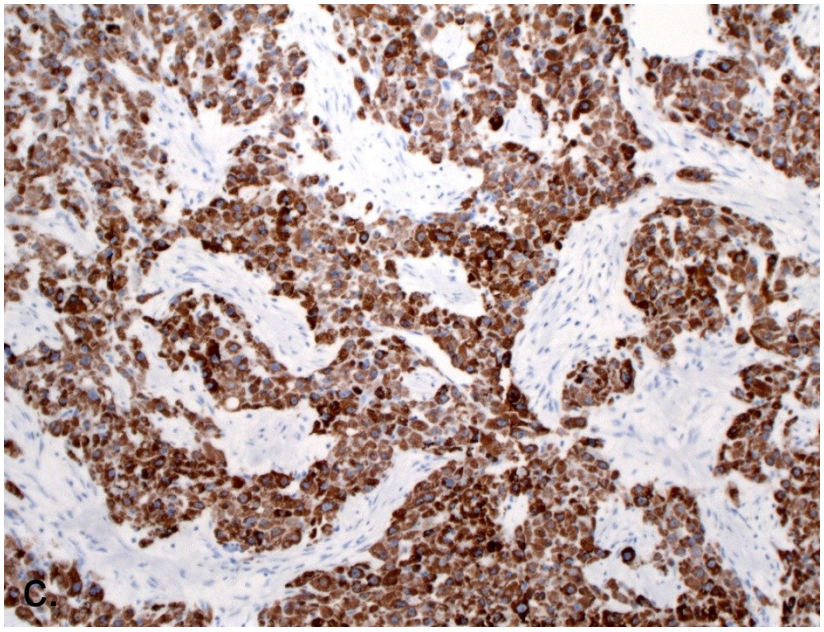


Figure 7







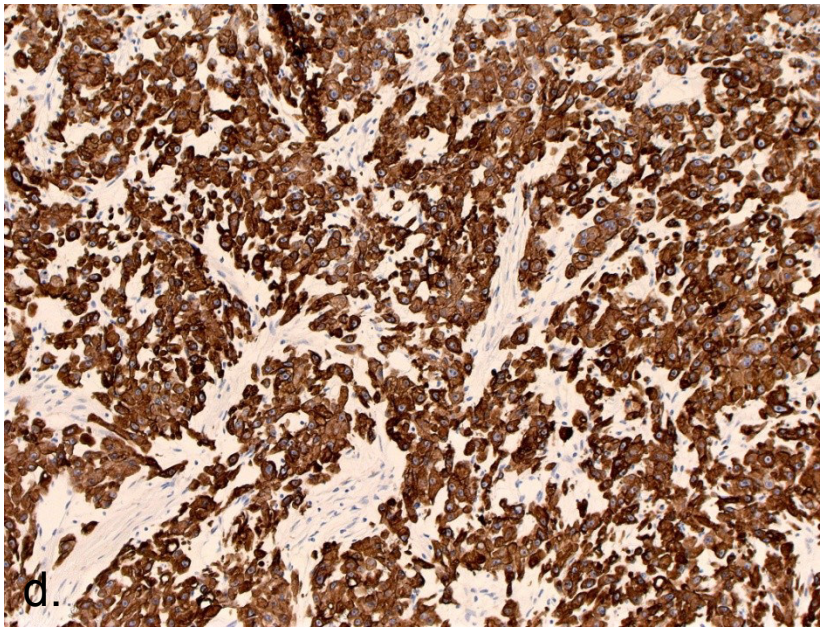
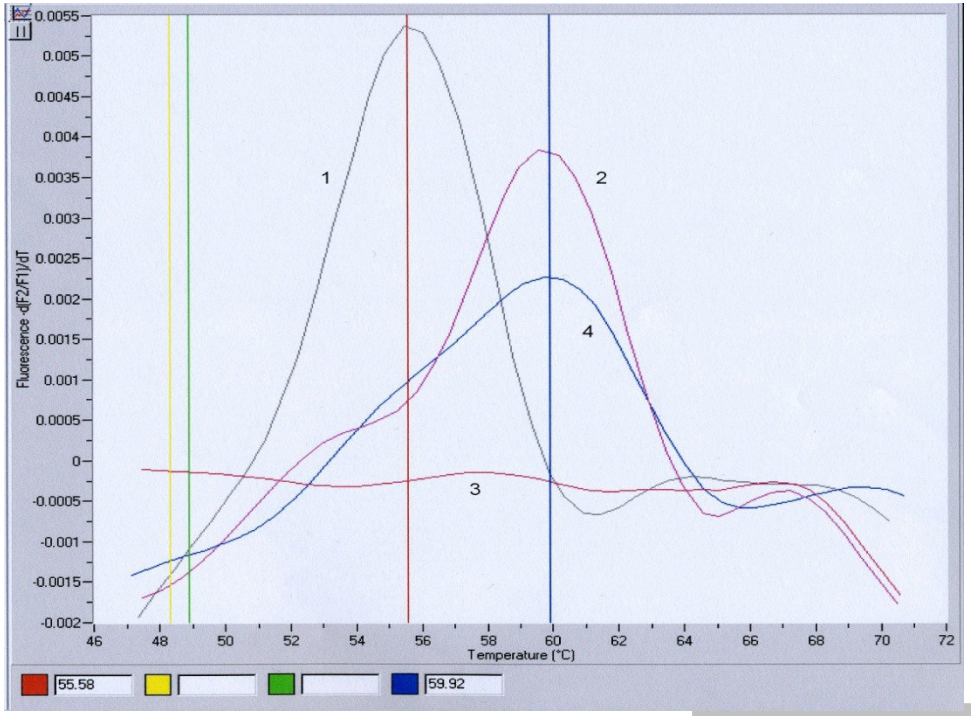
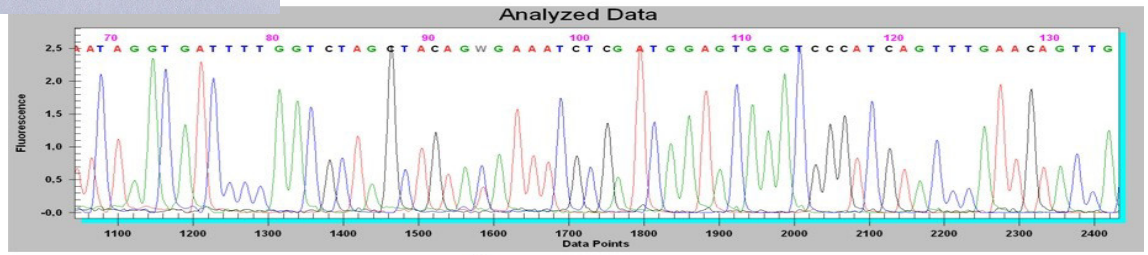


Figure 8





c.1799T>A
p.V600E

Region	Scope of study	Data source	Period covered	Overall	Men	Women	Study
Canada	National	Canadian cancer registry	1976-2000	-	6.0 (1996-2000)	2.0 (1996-2000)	Pocobelli et al.
		National and provincial cancer registries	1984-2000	-	5.5 (2000)	2.2 (2000)	Dyer et al.
USA	National	SEER 9 cancer registries	1982-1994	-	3 (1992-1994)	<1 (1992-1994)	Capocaccia et al.
		SEER cancer registries and CDC NPCR	1998-2003	-	6.2 (2003)	1.7 (2003)	Ahmed et al.
		SEER 9 and 13 cancer registries	1975-2005	4.9 (2005)	7.9 (2005)	2.3 (2005)	Altekurse et al.
Europe	Northern α	EUROCARE cancer registries	1983-1994	-	3 (1992-1994)	<1 (1992-1994)	Capocaccia et al.
	Southern b			-	12 (1992-1994)	3 (1992-1994)	
Netherlands	National	Netherlands cancer registry	1989-2000	-	1.6 (2000)	0.3 (2000)	Verhoef et al.
France	Regional (Bas-Rhin)	Single cancer registry	1990-1999	10.8 (1998-1999)	-	-	Binder-Foucard et al.
	Regional (Finiste're)	Progressive registration of cases by physicians	2002-2003	-	13.8 (2002-2003)	0.8 (2002-2003)	Caumes et al.
Japan	Regional (Osaka)	Osaka cancer registry	1990-2003	-	24.0 (2003)	7.3 (2003)	Tanaka et al.

Table 1. Studies reporting the age-standardized incidence of hepatocellular carcinoma in the general population.

Most recent estimate of age-standardized HCC incidence per 100.000 people (yrs).

Relevant studies published in the period between June 11, 2004 and June 11, 2009 were identified by searches of the U.S. National Library of Medicine PubMed database.
α Northern Europe = Denmark, Norway, Sweden, Iceland, and the UK.
b Southern Europe = Italy, France, and Spain.

Abbreviations: CDC NPCR, Centers for Disease Control and Prevention National Program of Cancer registries; SEER, Surveillance, Epidemiolgy and End results.

Table 2

Chronic hepatic injury (60%–90%)
Cirrhosis (most common)
Chronic hepatitis only (far less common) (HBV >> HCV)
Specific etiologies
High rate of associated HCC (>15%)
HBV[a]
HCV[a]
Hereditary hemochromatosis
Hereditary tyrosinemia
Porphyria cutanea tarda
Hypercitrulinemia[b]
Membranous obstruction of the inferior vena cava
Intermediate rate of associated HCC (5%–15%)
Alcohol[a]
A1AT deficiency
Glycogen storage disease (types 1 and 3)[b]
Autoimmune hepatitis (?)
Low rate to rare presence of associated HCC (<5%)
Primary biliary cirrhosis
Primary sclerosing cholangitis
Hereditary fructose intolerance[b]
Paucity of intrahepatic bile ducts[b]
Progressive intrahepatic cholestasis (Byler disease)
Congenital hepatic fibrosis
Biliary atresia
Wilson disease
Oral contraceptive steroids[b]
Anabolic-androgenic steroids[b,c]
Obesity
Diabetes mellitus
Cardiac cirrhosis
Exposure to various chemicals/ toxins, including aflatoxinB1[d]

[a] Most important specific etiologies associated with HCC worldwide. Multiple factors may act in a synergistic fashion, most frequently associated with cirrhosis.

[b] Conditions where HCC uniformly occurs in a noncirrhotic liver. Occasionally other conditions, such as chronic hepatitis B or alpha-1-antitrypsin deficiency or, rarely, chronic hepatitis C, alcoholic liver disease, or hereditary hemochromatosis, will lead to HCC in the absence of cirrhosis.

[c] Although hepatic tumors associated with anabolic-androgenic steroid usage may have the histologic appearance of HCC, biologically malignant behavior (metastasis) is rare.

[d] Although aflatoxin B1 has been strongly associated with the occurrence of HCC in regions of high incidence and in experimental animals, its role as a carcinogen appears to be synergistic with hepatitis B. Chronic exposure to vinyl chloride, pesticides/ herbicides, and other organic chemicals has occasionally been reported in association with HCC. Cigarette smoking has shown inconsistent association with HCC.

Table courtesy of Scott Saul, MD, Chester County Hospital, West Chester, PA.

Table 3: Histologic Grading of Hepatocellular Carcinoma

Grade	Architecture	Nuclear Features	Cytoplasmic Features/Cell Size	Other
Well-differentiated (grades I/II of Edmonson and Steiner)	Thin plates, 3 or fewer hepatocytes thick; pseudoglandular architecture common.	Minimal nuclear atypia; nuclear density greater than twice that of nonneoplastic liver.	Fatty change common. Tumor cells typically smaller than nonneoplastic cells.	Clear-cut histologic distinction from hepatocellular adenoma may not be possible in some cases without finding other, more poorly differentiated foci and knowing the status of the nonneoplastic liver. This pattern is typical of small (<2 cm) HCC.
Moderately differentiated (grades II/III of Edmonson and Steiner)	Trabecular pattern typical; plates more than 3 cells thick.	Nuclear atypia more pronounced.	Tumor cells are larger and have more abundant eosinophilic cytoplasm and distinct nucleoli, compared with well-differentiated tumors. Giant cells may be present. Bile may be seen.	Most common type of differentiation seen in advanced (>2 cm) HCC.
Poorly differentiated (grades III/IV of Edmonson and Steiner)	Compact growth pattern with rare or no trabeculae is common.	Pronounced nuclear atypia, enlargement, and hyperchromasia.	Bile is not present. Spindle or small cell areas may be seen.	May be difficult to recognize as hepatocellular in origin.

Modified from: Hamilton SR, Aaltonen LA, eds. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Digestive System. Lyon, France: IARC Press, 2000; and Yano Y, Yamamoto J, Kosuge T, et al. Combined hepatocellular and cholangiocarcinoma: a clinicopathologic study of 26 resected cases. Jpn J Clin Oncol 2003; 33(6): 283-287. Table courtesy of Scott Saul, MD, Chester County Hospital, West Chester, PA.

Table 4: Hepatocellular Carcinoma—Cytoplasmic Deposits and Inclusions [a]

Deposit/ Inclusion	Sensitivity (%)	Comments
Diagnostically useful	100	Mucin may be present in combined HCC-CCC, CCC, or metastatic adenocarcinoma
Absence of cytoplasmic mucin	5–33	Virtually pathognomonic of HCC
Bile	7–41	To date, negative in CCC and metastatic adenocarcinoma
Copper/ copper-binding protein	2–25	In malignant neoplasm, virtually pathognomonic of HCC
Mallory hyaline Hyaline globules	10–15	Highly suggestive of HCC in malignant hepatic tumor, but metastatic adenocarcinoma and neuroendocrine carcinoma may demonstrate these deposits
PAS-positive DR		AFP, A1AT, A1ACT, giant lysosomes, other glycoproteins
PAS-negative		Megamitochondria, apoptotic bodies, albumin, fibrinogen, other proteins
Ground-glass/ pale bodies	5–10	Fibrinogen, other serum proteins; HBsAg (usually represents trapped nonneoplastic cells)
Of interest, but not diagnostically useful	20–40	Predominant in 5%–16% of cases
Fat, glycogen (clear cells)	Rare, trace	True even in HCC arising in hereditary hemochromatosis
Hemosiderin	amounts	When prominent, liver may be black (Dubin-Johnson–like)
Lipofuscin-like pigment	Rare	

AFP, alpha-fetoprotein; A1AT, alpha1-antitrypsin; A1ACT, alpha1-antichymotrypsin; CCC, cholangiocarcinoma; DR, diastase-resistant; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; PAS, periodic acid-Schiff.

[a] Data primarily from: Ishak KG, Goodman ZD, Stocker JT, eds. Tumors of the Liver and Intrahepatic Bile Ducts. Washington, DC: Armed Forces Institute of Pathology, 2001; and Nzeako UC, Goodman ZD, Ishak KG. Hepatocellular carcinoma in cirrhotic and noncirrhotic livers: a clinicopathologic study of 804 North American patients. Am J Clin Path 1996; 105: 65-75. Table courtesy of Scott Saul, MD, Chester County Hospital, West Chester, PA.

Table 5: pTNM Staging of Primary Hepatic Epithelial Malignancies

HCC
T-- Primary tumor:
TX The primary tumor cannot be evaluated
T0 There is no evidence of a primary tumor

T1 Solitary tumor without vascular invasion
T2 Solitary tumor with vascular invasion *or* multiple tumors, no one bigger than 5 cm in maximal diameter
T3 Multiple tumors bigger than 5 cm in maximal diameter *or* tumors involve branches of portal or hepatic veins.
T3a Multiple tumors bigger than 5 cm in maximal diameter
T3b Tumor involves the branches of portal or hepatic veins
T4 Tumor (s) with direct invasion of the nearby organs (except the gallbladder) *or* tumor (s) with perforation of the visceral peritoneum

N-- Regional lymph nodes:
NX The regional lymph nodes cannot be evaluated
N0 Cancer has not spread to the regional lymph nodes
N1 The cancer has spread to the regional lymph nodes

M-- Distant metastases:
M0 No distant metastases
M1 Distant metastases present

Stage groupings:

Stage I	T1, N0, M0
Stage II	T2, N0, M0
Stage IIIA	T3a, N0, M0
Stage IIIB	T3b, N0, M0
Stage IIIC	T4, N0, M0
Stage IVA	Any T, N1, M0
Stage IVB	Any T, any N, M1

Intrahepatic bile ducts (CCC):
T-- Primary tumor:
TX The primary tumor cannot be evaluated
T0 There is no evidence of a primary tumor
Tis Carcinoma *in situ* (intraductal tumor)

T1 Solitary tumor without vascular invasion
T2a Solitary tumor with vascular invasion
T2b Multiple tumors with or without vascular invasion
T3 Tumor (s) with perforation of the visceral peritoneum *or* with direct invasion of extrahepatic structures
T4 Tumor with periductal invasion

N-- Regional lymph nodes:
NX The regional lymph nodes cannot be evaluated
N0 Cancer has not spread to the regional lymph nodes
N1 The cancer has spread to the regional lymph nodes

M-- Distant metastases:
M0 No distant metastases
M1 Distant metastases present

Stage groupings:

Stage I	T1, N0, M0
Stage II	T2, N0, M0
Stage III	T3, N0, M0
Stage IVA	T4, N0, M0
	Any T, N1, M0
Stage IVB	Any T, any N, M1

Perihilar bile ducts:
T-- Primary tumor:
TX The primary tumor cannot be evaluated
T0 There is no evidence of a primary tumor
Tis Carcinoma *in situ*

T1 Tumor confined to the bile ducts and invades the muscularis propria or the fibromuscular layer
T2a Tumor infiltrates beyond the bile ducts in the nearby soft tissue
T2b Tumor infiltrates the nearby liver
T3 Tumor infiltrates unilateral branches of portal vein or common hepatic artery
T4 Tumor infiltrates the main branch or bilateral branches of the portal vein or common hepatic artery; *or* infiltrates unilateral branches of the bile ducts (2^{nd} level) with infiltration of the contralateral branches of the portal vein or common hepatic artery

N-- Regional lymph nodes:
NX The regional lymph nodes cannot be evaluated
N0 Cancer has not spread to the regional lymph nodes
N1 The cancer has spread to the regional lymph nodes (cystic duct, choledochal duct, hepatic artery and portal vein)

M-- Distant metastases:
M0 No distant metastases
M1 Distant metastases present

Stage groupings:
Stage 0 Tis, N0, M0
Stage I T1, N0, M0
Stage II T2a, T2b, N0, M0
Stage IIIA T3, N0, M0
Stage IIIB T1, T2, T3, N0, N1, M0
Stage IVA T4, any N, M0
Stage IVB Any T, any N, M1

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Table 6A

		HCC (51)	ICC (40)	Klatskin (7)	HCC/CCC (3)	FLC (8 cases/7 patients)
Clinico/pathological						
Demographic	Males/Females	45/6	18/22	3/4	2/1	4/3
	Age (mean, Year)	64.6	60.5	58.6	42	31.4
	tumor size (mean, cm)	6.2	6.6	3.9	5.1	13.6
	cirrhosis	29/51 (56.9%)	4/40 (10%)	0/7	3/3	0/7
Location	right lobe	17/45 (33.3%)	6/38 (15%)	0/7	2/3	
	left lobe	13/45 (25.5%)	11/38 (27.5%)	5/7	0/3	
	both 2°	15/45 (29.4%)	21/38 (52.5%)	2/7	1/3	
Tumor grade	Grade 1	8/51 (15.7%)	1/40 (2.5%)	1/7	0/3	0/8
	Grade 2	42/51 (82.4%)	28/40 (70%)	5/7	1/3	8/8
	Grade 3	1/51 (1.9%)	11/40 (27.5%)	1/7	2/3	0/8
Histologic subtypes HCC	trabecular	32/51 (62.7%)				
	pseudoglandular	16/51 (31.4%)				
	solid	3/51 (5.9%)				
Histologic subtypes ICC	conventional		35/40 (87.5%)	7/7		
	mucinous		2/40 (5%)	0/7		
	signet ring		2/40 (5%)	1/7		
	clear cell		1/40 (2.5%)	0/7		
Operation	total hepatectomy	11/51 (21.6%)	0/39	0/7	0/3	0/4
	partial hepatectomy	40/51 (78.4%)	39/39 (100%)	7/7	3/3	4/4
Margins of resection	R0/R1	44/7	18/16	2/4	3/0	3/1
	Rx	0/40	5/40	1/7	0/3	0/4
Nodal status	Lymphnode resection	13/51	26/39	6/7	3/3	4/4
	N0	13/13 (100%)	10/26 (38.5%)	3/6	2/3	1/4
	N1	0/13	16/26 (61.5%)	3/6	1/3	3/4
Lymphatic invasion	L1	4/51 (7.8%)	6/39 (15.4%)	1/7	1/3	1/4
Vascular invasion	V1	4/51 (7.8%)	5/39 (12.8%)	4/7	1/3	1/4
Stage	T1	22/51 (43.2%)	21/39 (35.8%)	0/7	1/3	1/4
	T2	15/51 (29.4%)	13/39 (33.3%)	4/7	2/3	0/4
	T3	12/51 (23.5%)	4/39 (10.3%)	2/7	0/3	1/4
	T4	2/51 (3.9%)	1/39 (2.6%)	1/7	0/3	2/4

Table 6B

		HCC	ICC	Klatskin	HCC/CCC	FLC	*P*-value
Immunohistochemistry	HepPar1	46/51 (90.2%)	2/40	0/7	3/3 in HCC	8/8	<0.0001 (discrimination HCC/CCC)
	Glypican3	50/51 (98%)	NA	NA	NA	NA	
	AFP	4/51 (7.8%)	0/40	0/7	0/3	0/8	0,027 (discrimination HCC/CCC)
	CK7	10/51 (19.6%)	38/40 (95%)	6/7	3/3 in CCC	7/8	<0.0001 (discrimination CCC/HCC)
	SALL4	4/51 (7.8%)	3/40 (7.5%)	0/7	2/3	0/8	
	FGG	5/51 (9.8%)	NA	NA	NA	8/8	<0.0001 (discrimination FLC/HCC)
	β-Catenin	10/51 (19.6%)	3/40 (7.5%)	0/7	0/3	0/8	
	Wnt1	48/51 (94.1%)	34/40 (85%)	7/7	2/3 in HCC	8/8	
	BRAF	0/51	0/40	0/7	0/3	0/8	
	P53	13/51 (25.5%)	34/40 (85%)	5/7	3/3	4/8	
	CD10	36/51 (70.6%)	0/40	NA	NA	NA	<0.0001 (discrimination HCC/CCC)
Molecular pathology	BRAF	4/50	0/31	0/7	1/3 in HCC	1/8	

Table 7

Case no.	Sex	Age (yr)	Diagnose/ Grade	Subtype	DI (cm)	T	N	L	V	R
01	M	69	HCC/G3	Solid+trabecular	7.5	T1	Nx	L0	V0	R0
02	M	68	HCC/G2	Trabecular (clear-cell v.)	13	T3a	Nx	L0	V0	R1
03	M	74	HCC/G2	Trabecular	13	T3b	Nx	L0	V1	R0
04	M	66	HCC/G2	Trabecular	10	T1	Nx	L0	V0	R0
05	M	65	HCC/G2	Trabecular+PG (fat droplets)	3.5	T3b	Nx	L0	V1	R1
06	M	75	HCC/G2	Trabecular	3.2	T1	Nx	L0	V0	R0
07	M	74	HCC/G2	Trabecular	12	T3a	Nx	L0	V0	R0
08	M	70	HCC/G2	Trabecular+PG	7.5	T3a	0/5	L0	V0	R0
09	M	70	HCC/G2	Trabecular+PG	15	T1	Nx	L0	V0	R0
10	M	63	HCC/G1	Trabecular	2.8	T2m	Nx	L0	V0	R0
11	M	56	HCC/G2	Trabecular+PG	16	T1	Nx	L0	V0	R0
12	W	64	HCC/G2	Trabecular (fat droplets)	5.2	T3b	Nx	L1	V1	R0
13	M	71	HCC/G2	Solid+PG (clear-cell v.)	3	T2m	Nx	L0	V0	R0
14	M	38	HCC/G2	Trabecular (clear-cell v.)	17.5	T3b	0/4	L0	V1	R0
15	M	63	HCC/G2	Trabecular+PG	11	T3b	Nx	L1	V1	R0
16	W	70	HCC/G1	Trabecular+PG	21	T3a	Nx	L0	V0	R1
17	M	62	HCC/G2	Trabecular	6.5	T2m	0/1	L0	V0	R0
18	M	62	HCC/G2	Solid+trabecular	?	T1	Nx	L0	V0	R1
19	W	16	HCC/G2	PG	11	T3b	0/1	L0	V2	R0
20	M	63	HCC/G2	Trabecular	6.3	T2	Nx	L0	V1	R0
21	M	64	HCC/G1	Trabecular	1.8	T1	Nx	L0	V0	R0
22	M	62	HCC/G1	Trabecular+PG	7	T4	Nx	L0	V0	R0
23	M	65	HCC/G1	Solid+PG (fat droplets)	3.5	T1	0/1	L0	V0	R0
24	M	58	HCC/G2	Trabecular	2.8	T1	Nx	L0	V0	R0
25	M	70	HCC/G2	Solid (clear-cell v.)	3.1	T1	Nx	L0	V0	R0
26	W	69	HCC/G2	Solid	5.2	T3b	Nx	L0	V1	R1
27	M	63	HCC/G1	Trabecular+PG	5	T1	Nx	L0	V0	R0
28	W	71	HCC/G2	Solid+trabecular (clear-cell V.)	7	T1	Nx	L0	V0	R0
29	M	72	HCC/G2	Solid+trabecular	4.5	T1	Nx	L0	V0	R0
30	M	71	HCC/G2	Solid+trabecular	6.2	T2	Nx	L0	V1	R0
31	M	69	HCC/G2	Trabecular+PG	3.7	T1	Nx	L0	V0	R0
32	M	60	HCC/G2	Trabecular (clear-cell v.)	3	T2	0/1	L1	V1	R0
33	M	67	HCC/G1-2	Trabecular (fat droplets)	1.8	T2m	0/1	L0	V0	R0
34	M	54	HCC/G2	Trabecular (clear-cell v.)	7	T3a	Nx	L0	V0	R0
35	M	66	HCC/G2	Solid+trabecular	7	T2m	Nx	L0	V1	R1
36	M	69	HCC/G2	Trabecular (fat droplets)	3.5	T2m	Nx	L0	V0	R0
37	M	63	HCC/G1-2	Solid+trabekulär (fat droplets)	1.9	T1	Nx	L0	V0	R0
38	M	68	HCC/G2	Trabecular+PG	1.7	T2m	Nx	L0	V0	R0
39	M	61	HCC/G2	Trabecular+PG (fat droplets)	1.5	T2m	0/1	L0	V0	R0
40	M	77	HCC/G2	Solid+PG (spindle-/clear-cell v.)	2.4	rT1	Nx	L0	V0	R0
41	M	75	HCC/G2	Trabecular	2.8	T2m	Nx	L0	V0	R0
42	M	64	HCC/G2	Trabecular	2	T1	0/3	L0	V0	R0

43	M	71	HCC/G2	Trabecular (clear-cell v.)	5.5	T1	Nx	L0	V0	R0
44	M	66	HCC/G2	Trabecular	1.4	T1	Nx	L0	V0	R0
45	M	44	HCC/G2	Trabecular	1.6	T2m	0/3	L0	V0	R0
46	M	69	HCC/G2	Solid+PG	11	T2	0/13	L1	V1	R0
47	M	76	HCC/G1	Solid (fat droplets)	1.7	T1	Nx	L0	V0	R0
48	M	70	HCC/G2	Trabecular	8.5	T4	Nx	L0	V0	R1
49	W	58	HCC/G2	Trabecular	3.5	yT1	Nx	L0	V0	R0
50	M	56	HCC/G2	Trabecular	1.5	T1	0/1	L0	V0	R0
51	M	66	HCC/G1	Solid+trabecular	3.5	T2m	0/1	L0	V0	R0
52	M	70	ICC/G2	Conventional	2.5	T1	Nx	L0	V0	R0
53	M	63	ICC/G2	Conventional	10	T1	Nx	L0	V0	Rx
54	M	73	ICC/G2	Conventional	1.9	T1	Nx	L0	V0	R0
55	W	55	ICC/G2	Conventional	5.7	T1	Nx	L0	V0	Rx
56	W	47	ICC/G2	Conventional	8.2	T2bm	Nx	L1	V1	R0
57	W	64	ICC/G3	Conventional	5	T3	3/12	L1	V0	R1
58	M	65	ICC/G2	Conventional	4.4	T1	Nx	L0	V0	R0
59	M	77	ICC/G2	Conventional	7.2	T1	2/9	L0	V0	R0
60	M	62	ICC/G2	Conventional	4.5	T1	Nx	L0	V0	R0
61	W	64	ICC/G2	Conventional	3.5	T2a	Nx	L0	V 1	R0
62	W	46	ICC/G2	Mucinous	7.3	T2b	4/18	L0	V0	R0
63	W	53	ICC/G2	Conventional	10.7	T1	0/16	L0	V0	R0
64	M	63	ICC/G2	Conventional	3.8	T1	0/2	L0	V0	R1
65	M	60	ICC/G2	Conventional	3.5	T2b	Nx	L1	V1	Rx
66	W	52	ICC/G2	Conventional	4	T1	Nx	L0	V0	R0
67	M	68	ICC/G3	Conventional	0.7	T1	N1?	L0	V0	R1
68	W	59	ICC/G3	Conventional	?	T1	3/9	L0	V0	R0
69	M	58	ICC/G2	Conventional	6	T2b	1/1	L0	V0	R1
70	M	50	ICC/G3	Conventional	11	T3m	Nx	L0	V0	R0
71	W	60	ICC/G2	Conventional + clear-cell	8.5	T2bm	Nx	L1	V0	R0
72	M	55	ICC/G3	Conventional			9/9			
73	W	63	ICC/G2	Conventional	11.5	T2a	0/2	L0	V2	R1
74	W	58	ICC/G2	Conventional	11.5	T4	3/4	L0	V0	Rx
75	M	60	ICC/G2	Conventional	11.5	T2bm	1/14	L1	V0	R1
76	M	65	ICC/G3	Conventional + signet ring	6.5	T1	3/13	L0	V0	R1
77	M	72	ICC/G2	Conventional	5.2	T3	1/8	L0	V0	R1
78	M	78	ICC/G1	Conventional	6.2	T1	0/6	L0	V0	R0
79	W	54	ICC/G2	Conventional	3	T3	Nx	L0	V0	R0
80	W	66	ICC/G2	Conventional	8.2	T1	0/2	L0	V0	R0
81	W	50	ICC/G2	Mucinous	3.5	yT1	0/1	L0	V0	R1
82	W	57	ICC/G3	Conventional	11	T2b	0/3	L0	V0	R0
83	W	56	ICC/G3	Conventional	11.5	T1	1/20	L0	V0	R1
84	W	50	ICC/G3	Conventional	11	T1	0/11	L0	V0	R1
85	W	72	ICC/G3	Conventional	4.2	T2b	1/19	L0	V0	Rx
86	M	62	ICC/G3	Conventional	2.5	T1	Nx	L0	V0	R0
87	W	69	ICC/G2	Conventional	12	T2a	1/1	L1	V1	R1
88	W	77	ICC/G2	Conventional	7	T1	0/3	L0	V0	R1

89	M	57	ICC/G2	Conventional	5.5	T2bm	1/5	L0	V0	R1
90	W	48	ICC/G2	Conventional	5	T1	3/6	L0	V0	R1
91	W	42	ICC/G2	Conventional	7.2	T2b	0/1	L0	V0	R1
92	W	64	Klatskin/G2	Conventional	3	T2a	0/25	L0	V0	R0
93	M	50	Klatskin/G2	Conventional	4.3	T4	2/8	L0	V1	Rx
94	W	66	Klatskin/G2	Conventional	1.5	T2b	3/15	L0	V0	R0
95	M	57	Klatskin/G2	Conventional + signet ring	10	T3	0/4	L0	V2	R1
96	W	46	Klatskin/G1	Conventional	3	T2a	0/8	L0	V0	R1
97	W	69	Klatskin/G3	Conventional	1.8	T3	Nx	L0	V1	R1
98	M	58	Klatskin/G2	Conventional	?	T2b	8/23	L1	V1	R1
99	M	46	HCC/CCC-G3		6.5	T2a	12/14	L1	V0	R0
100	M	66	HCC/CCC-G2		1.8	T1	0/8	L0	V0	R0
101	W	14	HCC/CCC-G3		7	T2a	N0?	L0	V1	R0
102	M	17	FLC		14	T1	1/6	L0	V0	R0
103	W	39	FLC		14	T3b	2/11	L1	V0	R0
104	M	32	FLC		12	T4	0/3	L0	V0	R0
105	M	21	FLC		14.5	T4	2/10	L0	V0	R1
106	W	27	FLC							
107	W	18	FLC							
108	M	66	FLC							

Table 8						
Case No.	**Diagnosis**	**Recurrence/ Time to recurrence (months)**	**Metastases**	**Follow up (months)**	**Clinical history**	**Death date/ Time to death (months)/ Cause**
01	HCC	No	Lymphnodes	6	HCV-cirrhosis	
02	HCC	No		0		
03	HCC	No		1		
04	HCC	No		47	cirrhosis (alcohol)	
05	HCC	No		0		
06	HCC	No		31	rectal ca 01.2007	
07	HCC	No	peritoneal	26		
08	HCC	Yes, 11		11	HCV	
09	HCC	Yes, 11	bone/soft tisse	11		
10	HCC	No		42		
11	HCC	Yes, 4	multiorgan	9		
12	HCC	No		0		
13	HCC	Yes, 16		40	rectal ca and liver mets 06.2008	
14	HCC	No		0	Hemochromatosis	
15	HCC	Yes, 11		11		
16	HCC	Yes, 25		34	cryptogenic liver fibrosis	
17	HCC	No		3		
18	HCC	No		10		
19	HCC	No		16		
20	HCC	No		NA		
21	HCC	No		0		
22	HCC	No		0		
23	HCC	Yes, 6		27	Oesophagus ca 2007	
24	HCC	No		26	HBV	
25	HCC	No		25		
26	HCC	No		NA		
27	HCC	No		NA		
28	HCC	No		78		
29	HCC	No		NA		
30	HCC	No		NA		
31	HCC	No		0		
32	HCC	Yes, 21		21	HBV	
33	HCC	No		33	HCV	

34	HCC	No		32	HCV	Yes, 32, re-cirrhosis
35	HCC	Yes, 10		43		
36	HCC	No		16	HCV	
37	HCC	No		67		
38	HCC	No		3		
39	HCC	No		58	Prostate ca 03.2010	
40	HCC	No	bone	13		
41	HCC	Yes, 16	peritoneal carcinomatosis	26		
42	HCC	No		42		
43	HCC	No		27	NHL 04.2009	
44	HCC	Yes, 8		35	HCV	
45	HCC	No		33	HBV	
46	HCC	No		17		
47	HCC	No		28	colon ca 02.2007	
48	HCC	No		16		
49	HCC	No		30	HBV	
50	HCC	No		24	Wilson/ liver fibrosis	
51	HCC	No		23	cryptogenic cirrhosis	
52	ICC	No		NA		
53	ICC	No		NA		
54	ICC	No		NA		
55	ICC	No		NA		
56	ICC	No		NA		
57	ICC	No		NA		
58	ICC	Yes, 17		42		
59	ICC	No	Lymohnodes	9		
60	ICC	No		NA		
61	ICC	No		81		
62	ICC	Yes, 4		7		
63	ICC	No		1		
64	ICC	Yes, 45		62	Melanoma	Yes, 62
65	ICC	Yes, 10		23		
66	ICC	Yes, 61	Omentum	61		
67	ICC	No		NA		
68	ICC	No		NA		
69	ICC	No	peritoneal carcinomatosis	NA		
70	ICC	No		NA		

71	ICC	No		NA	NASH	
72	ICC	No		NA	HBV and fibrosis	
73	ICC	Yes, 25	Lymphnodes (mediast/cervical), Lung	34		Yes, 34
74	ICC	No	peritoneal carcinomatosis	7		
75	ICC	No	peritoneal	7		
76	ICC	No		0		
77	ICC	No	single bone mets	8		
78	ICC	No		10		Yes, 10, multiorgan failure
79	ICC	No		23		
80	ICC	No		NA		
81	ICC	No		21		Yes, 21
82	ICC	No		1	IDC 2010	
83	ICC	Yes, 10		22		
84	ICC	No		22		
85	ICC	No	paravertebral/ pleural,	9		
86	ICC	No		19	HBV+cirrhosis	
87	ICC	No		1		
88	ICC	No		15		
89	ICC	No		9		
90	ICC	No		1		
91	ICC	Yes, 5		8		
92	Klatskin	Yes, 69		69		
93	Klatskin	Yes, 5		7		Yes, 7, multiorgan failure
94	Klatskin	No		44		
95	Klatskin	No		33		
96	Klatskin	Yes, 16	peritoneal carcinomatosis,	27		
97	Klatskin	No		9	AML/NHL	Yes, 9, AML-recurrence
98	Klatskin	No		1	HBV/HCV	
99	HCC/CCC	Yes, 15		17		
100	HCC/CCC	Yes, 32		32	NHL	
101	HCC/CCC	Yes, 3	abdominal wall	3		
102	FLC	Yes, 15		90		
103	FLC	No	Lymphnodes retropancreatic	67		

104	FLC	No	Lymphnodes	19		
105	FLC	No	bone, lung, peritoneum	2		
106	FLC	No		NA		
107	FLC	No		NA		
108	FLC	No		NA		

Table 9

Tumor	Biomarker	Correlation and *P*-value
HCC		
	P53	higher tumor grade (P=0.0019)
	β-Catenin	Wnt1 expression (P=0.034)
	Wnt1	loss of P53 expression (P=0.012)
	SALL4	higher tumor grade (P=0.040)
		higher pT stage (P=0.035)
		vascular invasion (P=0.022)
		higher serum AFP levels (P=0.026)
		loss of Wnt1 expression (P<0.0001)
	BRAF-V600E	higher tumor grade (P=0.040),
		tumor localization in the both hepatic lobes (P=0.40)
		positive serology for HCV and HBV (P=0.038)
CCC		
	P53	higher tumor grade (P=0.0052)
	β-Catenin	vascular invasion (P=0.048)
		tumor localization in the left hepatic lobe (P=0.018)
	SALL4	multiple tumor nodules (P=0.0035)
		vascular invasion (P=0.048)
		higher recurrence rate (P=0.035)

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