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Advances in Diagnosis and Treatment

Edited by Costin Teodor Streba,
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HEPATOCELLULAR CARCINOMA - ADVANCES IN DIAGNOSIS AND TREATMENT

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Meet the editors

Costin Teodor Streba, MD, PhD, MSc, is an Associate Professor at the University of Medicine and Pharmacy of Craiova. He is a member of the Research Center of Gastroenterology and Hepatology of Craiova, within the same university. He has undertaken research in the field of liver and pancreatic disease diagnostics, while also receiving extensive training in histological image analysis. He specialized in devising medical-oriented diagnostic systems for liver malignancies, which integrate interpretation and computer-aided quantification of various imaging and clinical data. His publication record reflect his dedication for innovative diagnostic techniques in gastroenterology, having numerous large-scale studies featured in various high-impact publications within this field.

Cristin Constantin Vere, MD, PhD, MSc, is a Professor of Gastroenterology at the University of Medicine and Pharmacy of Craiova and one of the founding members of the Research Center of Gastroenterology and Hepatology of Craiova. His research interests span from neuroimmune mechanisms of liver disease to novel diagnostic techniques in gastroenterology. Pioneering the introduction of wireless videocapsule in Romania, he established a dynamic team of PhD students and full-time scientists that specialized in both medical sciences and bioinformatics. His publication record attests to his dedication for innovation in the field of hepatology and the complex integrative mechanisms that form the basis for this pathology.

Ion Rogoveanu, MD, PhD, MSc, is a Professor of Gastroenterology at the University of Medicine and Pharmacy of Craiova and one of the founding members of the Research Center of Gastroenterology and Hepatology of Craiova. He mentors a team of dedicated doctors and manages the curricular and scientific activities of the University as Rector. He is currently one of the lead authorities in Ultrasound and Power Doppler US, coordinating postgraduate courses for the past 10 years. His most prestigious recent publications are all in the field of prevention, diagnosis and treatment of HCC and various liver diseases.

Contents

Preface

The field of cancer research is one of the most dynamic ones in today's medical world. From screening and early diagnosis to deciphering novel molecular pathways and innovative surgical solutions, the ongoing battle against malignancies seems to progress to new grounds. Since this "silent killer" became acknowledged as one of the leading causes of death worldwide, the landscape continuously changes. The incidence of some tumors has been on the rise, while mortality decreased in other cases. All this is due to new risk factors attributed to modern society on one hand, and more diagnostic capabilities leading to early detection coupled with more effective therapeutic options, on the other hand.

In this ever-changing context, hepatocellular carcinoma (HCC) is a very interesting culprit. One of the few malignancies with an almost omnipresent association with previous pathology of the same organ, HCC was long thought to provide little surprises. However, since dietary habits and heavy industrialization led to new types of liver injury, what was once thought of as the only certain "at risk" population is now far more diverse. Endemic areas slowly but surely shift, along with modern efficient treatment options for viral hepatitis and the rise of obesity and metabolic disorders – that quietly place other population groups in top positions as HCC candidates.

Current guidelines suffered incremental changes over the last years, as imaging took the leading role in diagnosis, with the advent of contrast agents that can accurately characterize liver lesions. Recent acknowledgment of non-invasive, minimum risk investigations such as contrast-enhanced ultrasound already means better diagnostic options in a variety of novel settings, and for even more categories of patients compared to just a few years ago. As modern imaging methods become more available at decreased costs – thus being available in even smaller medical centers, the expertise of clinicians on all continents gradually increases, leading to more cases of early HCC diagnosed (and, thus, candidates for curative surgery).

Oncological treatment for HCC has been the subject of long academic debates in recent years. With limited options available, due to a small number of targetable cellular and molecular pathways, it seemed a lost battle; however, recent research proves the contrary. In recent years we have witnessed surprising breakthroughs, new lines of therapy, perhaps even a shift in paradigm in regards to invasive loco-regional treatment options. More efficient targeted drug-delivery methods, coupled with minimally invasive trans-arterial catheter-based solutions, may offer a broader array of options for intermediate stages and otherwise untreatable HCCs. Surgery has become more refined, more nuanced in its therapeutic options and goals. Novel techniques, based on minimally invasive set-ups, offer increased survivability at a lower time and anatomic cost for the patient.

With all these prospects in mind, we feel that this book project comes at the right time – when we are on the "high-tide" of HCC management, having authors that present recent breakthroughs, as well as re-establishing core-concepts and revising the basic concepts of this malignancy. We hope that everyone can find something of interest in this current volume – the goal was to bring together as many views and perspectives as possible, in a coherent, easy to follow, format.

We would like to extend our immense gratitude towards our mentors, close and distant collaborators that offered their invaluable contribution to our daily practice and academic efforts. Also, we would like to thank the authors, their collaborators, as well as the editorial team that made this project possible. A final – and most important – "thank you" goes to our families who give us the motivation to go forward and always offer their unconditional support.

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Introduction

Chapter 1

Introductory Chapter: Etiology and Pathogenesis of Hepatocellular Carcinoma

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Additional information is available at the end of the chapter

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1. Introduction

Hepatocellular carcinoma (HCC) is the most frequent malignant tumor of the liver with hundreds of thousands of new cases diagnosed each year. Men are up to 3 times more likely to develop HCC compared to women. HCC encounters a higher incidence in countries with low socio-economic status and with improper access to healthcare. These countries also associate high alcohol intake among the population as well as increased incidence of hepatotropic viruses or human immunodeficiency virus (HIV). On the other hand, screening and surveillance of patients at risk have determined the upturn of survivability in HCC patients.

2. Risk factors

HCC has several well-known risk factors, which have been proven to strongly associate with the development of HCC. The most common etiological risk factors are hepatotropic viruses: hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV) and a suggestive evidence is revealed by similar distribution of HCC in areas where these viruses also encounter increasing incidence and it is considered that up to 90% of the diagnosed HCCs develop in context of hidden cirrhosis [1, 2]. Other risk factors that are highly involved in the hepatocellular carcinogenesis also include autoimmune hepatitis, nonalcoholic fatty liver disease (NAFLD), obesity and diabetes, tobacco and alcohol abuse, environmental toxins, and iron overload.

2.1. Cirrhosis

Cirrhosis is the main underlying cause for most HCC cases, with HBV, and HCV infection often involved in the development of cirrhosis. Approximately 70–90% of liver cancers occur on cirrhosis, and in Western countries, the HCC ratio on cirrhosis exceeds 90%. The likelihood of developing HCC in viral B cirrhosis is 2.4% per year, and viral C cirrhosis is 5–7% per year. In Europe, HCC incidence is 1.5–3/100 cirrhosis per year. Male gender, advanced age, long duration of the disease and the severity of the disease are the main risk factors for developing cancer in cirrhosis alongside etiology of cirrhosis [3].

The progression from cirrhosis to HCC is a complex process. Cirrhosis is the outcome of any chronic hepatic illness and it is outlined by debilitation of regenerative capacity of the liver through declining proliferation of the hepatocytes [4]. Telomere dysfunction and alterations of cellular micro- and macroenvironment have been proven to enhance cellular proliferation [5]. Telomerase dysfunctions determine chromosomal instability and reduced regenerative liver capacity with decreased hepatocyte regeneration. It has been proven that telomeres are shorter in hepatocytes from a cirrhotic liver compared to a normal liver. Also, shorter telomeres are associated with the progression of liver fibrosis [6].

Several mouse models studies have suggested that telomerase dysfunctions have been associated with early-stage liver cancers but not with high-grade HCCs, which tends to indicate that telomere dysfunction cannot determine alone the development and progression of HCC in cirrhotic livers [7]. Van Gijssel et al. supported this idea by using a rat model in which they decreased hepatocyte proliferation with various hepatotoxic compounds that also increased carcinogen-induced tumor forming [8]. Activation of stellate cells in liver cirrhosis can increase products of oxidative stress, several growth factors as well as cytokines with further roles in reducing hepatocyte regeneration, and development of HCC [9]. Outbreaks of dysplasia occur in regeneration nodules, followed by neoplastic transformation. HCC rarely develops on the noncytotoxic liver and this is particularly common in HBV infection, hemochromatosis or HCV infection. The existence of viral infection or portal hypertension can increase the odds of developing HCC for patients with primary biliary cirrhosis [10].

2.2. HBV infection

HBV is regarded as the main etiological factor that generates multiple pathological changes inside the liver structure, being responsible for the development of HCC over time [11]. However, in order to correctly assess the risk of carcinogenesis triggered by chronic HBV infection, multiple variables need to be considered, like a virus or host-related factors and also the patient's lifestyle [12]. A major study published Chen CJ et al. evaluated the risk of developing HCC in 3653 patients who were positive HBV infection and negative for hepatitis C antibodies. The authors concluded that recorded serum levels of HBV DNA higher or equal to 10,000 copies/mL are a significant risk predictor for the development of HCC, no matter the Hepatitis B antigen level and liver cirrhosis [13].

In highly endemic regions, HBV is mainly transmitted from mother to child during birth (perinatal exposure). In developed countries, HBV infection is primarily contracted through parental contact with infected blood or through sexual contact [14]. Co-infection with HBV

is found in 9% HIV-infected patients, resulting in an increased risk of developing HCC compared to chronic HBV infection alone [15]. At the time of writing, there are 10 genotypes of human HBV named from A to J. The last genotype (J) was described in 2009 by Tatematsu K et al. [16], while the highest risk of developing HCC is linked with genotype C [17].

The prevalence of HBV carriers associates geographically with the distribution of HCC. Epidemiological studies indicated a 200-fold increase in HCC risk in Taiwanese HVB men compared to HBV-negative men [18]. Cirrhosis developed from chronic HBV infection is globally the most important etiologic factor of HCC.

Hepatocarcinogenesis generated by chronic HBV infection is a multistep process that implies rearrangement of the intracellular DNA leading to inflammation of the hepatocytes, accompanied by an increased rate of proliferation [19]. After the integration of viral DNA into the host's genome, the telomerase reverse transcriptase is altered and multiple genes involved in the malignant process suffer various insertional mutations [20]. If the inflammation process continues to affect the hepatocytes, the liver will respond to injury with necrosis of the affected areas, followed by compensatory regeneration and hepatic fibrosis, therefore, altering the entire hepatic architecture, leading to cirrhosis [21]. Recent studies enhance the importance of HBV X protein, suggesting that pathways like p38MAPK and PI-3 K/AKT are used in order to increase the invasive potential of HBV infection [22, 23]. The association of HBV infection with HCV or HVD or with increased alcohol intake or aflatoxin consumption increases the carcinogenic risk of HBV [24].

2.3. HCV infection

Chronic hepatitis C infection is a major risk factor for developing HCC. In developed countries, HCV is the important risk factor for HCC. HCV-associated HCC patients are usually significantly older than those with HCC associated with HBV infection [16].

The evolution over time of the viral infection in a few countries is pledged for the massive increase of HCC incidence. The major spread of HCV infection took place in Japan around the 1930s and in the US in the 1960s. These assessments are consistent with epidemiological observations and allow the estimate that HCC prevalence will increase in the US over the next 2–3 decades when it is likely to match that in Japan [25]. HBV co-infection, present in 3–13% of patients with viral hepatitis C, is associated with a HCC risk of 3–4 times the incidence of each infection [26]. It is considered that the survivors of the Hiroshima and Nagasaki nuclear bombs that were HCV-positive had a much higher risk of developing HCC in the absence of cirrhosis. It was suggested that the radiation had a mutagenic effect and C virus stimulated cell proliferation in these patients [27]. Almost all HCV-related hepatocarcinomas occur due to cirrhosis or chronic inflammation. It is therefore, believed that HCV is an indirect carcinogenic agent by induced inflammatory and necrotic lesions. Core protein influences various cellular functions, including apoptosis, and suppresses p53 activity [28–30].

2.4. Autoimmune hepatitis

The risk of developing HCC for patients with underlying autoimmune hepatitis still remains unclear. Development of HCC in the absence of cirrhosis or viral hepatitis is rather rare or

isolated [31]. A recent meta-analysis concluded that the risk of HCC is much lower for patients with autoimmune hepatitis and cirrhosis than for patients with cirrhosis from viral hepatitis or primary biliary cholangitis [32, 33]. Development of HCC from autoimmune hepatitis with corticosteroid-therapy should mainly impose searching for associated viral chronic hepatitis or any other HCC risk factors that can promote carcinogenesis [34].

2.5. Tobacco and alcohol abuse

Tobacco and alcohol abuse represent important HCC risk factors and exposure to both risk factors can increase HCC susceptibility. The mechanism involves generation of reactive oxygen species (ROS) and a decrease of antioxidants, which induces oxidative stress [35].

Alcohol chronic intake is associated with HCC development due to the several mechanisms such as creation of acetaldehyde-DNA; formation of cytochrome P450E1-associated ROS species; iron overload, which can lead to further ROS formation and p53 gene mutation or activation of factor-KappaB-involved in the promotion of inflammatory response; oxidative stress promotion; and decreased metabolism of vitamin A, which determines the promotion of hepatocyte proliferation as well as initiation and development of liver fibrosis [36]. Alcohol interferes with hepatocarcinogenesis by inducing an already demonstrated precancerous lesion, such as liver cirrhosis or by modifying carcinogenesis initiated by other agents such as HBV or HCV or environmental carcinogens following hepatic enzyme induction or by altering cell membranes [37].

2.6. Environmental toxins

Aflatoxin b1 derived from a fungus (Aspergillus flavus) is a major risk factor in some tropical and subtropical regions. Aspergillus flavus is ubiquitous and contaminates cereals (corn, rice, and sorghum), hazelnuts, etc., stored in humidity conditions. Epidemiological data have shown a strong correlation between aflatoxin intake and HCC incidence in some countries in Asia and Africa. Since 1993, the International Agency for Research on Cancer recognized aflatoxins as a human carcinogen (group IA) [38]. Advanced age, smoking, alcohol, and HBV infection may increase the carcinogenic risk of aflatoxin [39].

2.7. Obesity, diabetes and nonalcoholic fatty liver disease (NAFLD)

Obesity represents an important public health problem, with a massive increase in the past years and with staggering estimations of approximately 300 million obese worldwide. Obesity elevates the risk of all types of cancer, including HCC [40]. One study performed in Denmark on a cohort of 43.965 obese patients estimated the relative risk of liver cancer to 1.9 in comparison to the general population [41]. Two Swedish population-based cohort studies also showed an increased risk of HCC among obese [42, 43].

In another US study, Nair et al. evaluated the importance of obesity in over 19,000 patients diagnosed with cirrhosis and liver transplants, with an overall incidence for HCC of 3.5%. The study suggested obesity as a statistically independent risk factor for liver cancer in patients with alcoholic and cryptogenic cirrhosis [44]. Furthermore, a recent case–control study

indicated synergy between increased alcohol intake, smoking, and obesity [45]. In 2014, an American study regarding the incidence of hepatocellular carcinoma in Texas Latinos concluded that the incidence of liver cancer is somehow higher than other regions in the US, suggesting risk factors related to increased obesity and diabetes rates, as well as environmental, cultural and socioeconomic factors, and possibly genetic predisposition [46].

The mechanism by which obesity leads to cancer is unclear; insulin resistance and its subsequent inflammatory cascade, and insulin growth factor (IGF)-1 seem to be implicated [47]. In a study published in 2010, Michael Karin's team addressed the mechanism by which the obesity can lead to cancer by studying the development of HCC induced by diethylnitrosamine (DEN) or fat diet in mice [48, 49].

Although this is not entirely proven, a number of studies indicate that NAFLD is the link between obesity, diabetes, and HCC. In time, NAFLD can lead to fibrosis and finally, cirrhosis. Approximately 60% of patients with obesity have simple steatosis or steatosis with mild inflammation and around 25–30% have nonalcoholic steatohepatitis (NASH) [50].

Further mechanisms involved in the development of HCC at obese patients were addressed by Villanueva et al. by studying the molecular links between inflammation and liver cancer uncovering the reported role of lymphotoxin signaling in HCC development. The involvement of oxidative stress in developing HCC in obese patients was studied by Zhang, Kaufman et al., who underlined that the accumulation of intracellular lipids increases the demand on the endoplasmic reticulum (ER), which integrates several metabolic processes, therefore inducing ER dysfunction that leads to the production of ROS, provoking oxidative stress and activation of inflammatory pathways (NF-kB and JNK signaling). Another effect of oxidative stress is that can also induce DNA damage that leads to genomic instability that prompts the mutations that favor the development of neoplastic cells [51–53]. Carbohydrate metabolism alterations are frequently encountered at patients with cirrhosis [54].

Since 1986 at least 10 case-control and 5 prior cohort studies from seven different countries reported a connection between diabetes and HCC, promoting the idea that diabetes is an important and consistent risk factor for HCC [55–57]. However, the current studies have not established if diabetes precedes HCC.

The association among obesity, diabetes, NAFLD, and HCC has been assessed by El-Serag et al. in two large studies that substantiated the increased risk of HCC by obtaining results, which showed a doubling number of cases with HCC in patients with diabetes in contrast with nondiabetic patients in a 10–15 year observation period, explaining that the rising incidence of HCC in the US in the past 30 years is connected to an ever-growing prevalence of obesity and diabetes [58, 59].

Since the incidence of obesity and diabetes is in a continuous growth in the world, Kelly, and co. study demonstrated a direct established relationship between diabetes and HCC risk. The biological mechanism of diabetes implicated in hepatocarcinogenesis is not entirely established. Increased serum levels of insulin are at this point the most researched mechanism for the link between diabetes and cancer, though only high levels of insulin are not enough to cause HCC. Levels of insulin-like growth factor-1 (IGF-1) have been linked with increased

risk for pancreatic cancer [60–62]. Most studies indicate that serum IGF-1 levels were linked with the high-risk of HCC, and also that IGF-1 can promote tumor cell growth [63–66]. This was often linked to cell proliferation in pancreatic cancer and similar effects could be observed in HCC [62, 67].

As diabetes and obesity continue to be an ever-growing worldwide concern, we can anticipate a near future increase in the prevalence of NAFLD-related HCC [68]. If liver cirrhosis is present, NAFLD patients have a substantially higher risk to develop HCC [69]. Obesity is linked with a low-grade inflammatory status and also an increased production of cytokines like IL-6 or TNF-alpha [70]. Multiple potential carcinogenic mechanisms are also involved, such as reduced levels of adiponectin [71, 72], hepatic lipid accumulation with possible energy support required for massive tumor growth [73] or normal intracellular signaling means affected by lipotoxicity [74].

2.8. Iron overload

Almost two thirds of the total iron pool is present in hemoglobin while the rest of it is stored, mostly inside the liver, with the help of an intracellular protein called ferritin, which can bind up to 4500 molecules of iron per molecule of ferritin. Transferrin is a glycoprotein responsible for binding the circulating iron within the plasma [75]. Iron overload has been mainly associated with hereditary hemochromatosis (HH) and dietary iron overload (DIO).

Iron overload is frequently linked with an abnormal secretion of hepcidin [76, 77]. Recent studies performed on rats, which underwent a high-iron diet also confirm the possibility to develop HCC in the absence of liver cirrhosis, therefore, excessive iron is capable to generate oxidative tissue damage alone by accelerating the development of free radicals [78, 79]. DIO has been reported in some countries located in the southern and central part of Africa, mainly in the rural parts and highlights the link between the consumption of large volumes of home-brewed alcohol using iron containers, and development of iron overload [79].

3. Conclusion

HCC is a complex pathogenesis link with various risk factors. Liver cirrhosis is, unsurprisingly, an important risk factor for HCC development, regardless of the cause, whereas chronic HBV and HCV infections are the most significant developing factors for liver cancer worldwide. Therefore, frequent causes of cirrhosis are indicated as risk factors for HCC. The common factors affecting the progression to HCC in patients with cirrhosis are host and viral related with the involvement of external risk factors such as smoking, alcohol, and aflatoxins.

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Section 2

Diagnosis of Hepatocellular Carcinoma

Chapter 2

Diagnostic Algorithm of Hepatocellular Carcinoma: Classics and Innovations in Radiology and Pathology

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Additional information is available at the end of the chapter

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Abstract

In the global cancer statistics, hepatocellular carcinoma (HCC) ranges sixth by incidence and second by oncological mortality. The risk factors comprise hepatitis B and C virus infection, non-alcoholic steatohepatitis, as well as long-lasting peroral exposure to alcohol or aflatoxins. Liver cirrhosis is the most important single predisposing factor. Ultrasonography once per 6 months is recommended for surveillance in cirrhotic patients. Computed tomography (CT) and magnetic resonance imaging (MRI) represent the gold standard of non-invasive diagnostics while core biopsy and/or immunohistochemistry (IHC) are indicated for controversial and non-cirrhotic HCC cases. Molecular classification is under development. At present, classics of HCC diagnostics is based on evaluation of risk factors, surveillance in cirrhotic patients, preference for CT or MRI-confirmed non-invasive diagnosis and biopsy proof in equivocal cases. Diffusion-weighted imaging and hepatobiliary phase contrasting represent significant recent developments in MRI. Contrast-enhanced ultrasonography is recommended by some but not all guidelines. Positron emission tomography is advocated before liver transplantation to detect extrahepatic metastases but has limited role in the initial diagnostic evaluation of liver nodule. Innovations are expected in the field of molecular diagnostics, including IHC panels and novel antigens, e.g. clathrin and bile salt export pump protein, and development of molecular classification.

Keywords: hepatocellular carcinoma, HCC, diagnosis, imaging, CT, MRI, contrast-enhanced ultrasound, CEUS, positron emission tomography, PET, liver biopsy, immunohistochemistry

1. Introduction

Hepatocellular carcinoma (HCC) is a primary malignant liver tumour exhibiting hepatocellular differentiation [1]. It is well known for the strong association with preceding chronic liver disease and liver cirrhosis [2]. However, nowadays an increasing proportion of HCCs develops in non-fibrotic liver or on the background of mild fibrosis [3, 4]. The changing patterns of presentation influence the diagnostic approach both because of alterations in risk groups that could be targeted by surveillance and limits of non-invasive diagnostics in non-cirrhotic cases. In addition, the differential diagnostics of liver nodule differs in regard to the presence or absence of background liver cirrhosis. In cirrhotic liver, 59–94% (depending on size) of new mass lesions are malignant [5]. Thus, in patients with liver cirrhosis or preceding chronic liver disease, new nodule favours the diagnosis of HCC, as metastases and benign liver tumours are uncommon in cirrhotic liver [6, 7]. Hence, any mass lesion in cirrhotic liver must be considered HCC until proven otherwise [7].

In the global cancer statistics, HCC represents a frequent and aggressive tumour although different geographic regions face various burden of it. Worldwide, liver cancer is estimated to range sixth by incidence and second by oncological mortality causing 5.6% of global cancer incidence and 9.1% of mortality [8]. HCC is the most frequent primary liver cancer (>90%) being significantly more widespread than cholangiocarcinomas, hepatoblastomas and other primary liver malignancies [1]. Number of death cases per year (recently assessed by Ferlay et al. for the year 2012 as 745,000) is virtually identical to the incidence throughout the world (782,000; the same source) underlining the unfavourable course. The high ratio of mortality to incidence (0.95) reflects the dismal prognosis. As the geographical patterns of incidence and mortality closely follow each other [8], liver cancer is still an unsolved problem in the whole world.

According to the data provided by Surveillance, Epidemiology and End Results (SEER) Program of the National Cancer Institute, the 5-year survival for liver cancer is 16.6%, ranging from 30.5% in localised stage to 10.7% in regional stage and 3.1% in distant stage [2]. Different but similarly discouraging estimates have been reported, including 1-, 5- and 10-year survival of 31.3, 5.1 and 0.8%. The median survival is 6 months. However, significantly better outcome can be reached in early cases. Thus, median survival reaches 107 months in patients receiving liver transplantation for early HCC [9].

The incidence of liver cancer is high in Eastern and Southeastern Asia, followed by Northern and Western Africa [8]. China, Mongolia and Japan experience high occurrence [10]. In Europe, the highest age-standardised incidence rate of liver cancer is observed in Southern Europe [8] including Italy and France [10]. Although more developed regions generally show lower incidence of liver cancer (except Japan, France and Italy), its incidence is growing in many countries [8, 10]. Thus, although the total cancer incidence in the United States of America (USA) decreased in males and remained stable in females over time period 2003–2012, liver cancer incidence rates increased in both genders: 3.7% yearly in males and 3.0% in females. According to the National Program of Cancer Registries and SEER database, liver cancer incidence rate (2008–2012) in USA has increased by 2.3% per year [2]. The incidence rate of

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histologically proved HCC in USA has increased from 1.4/100,000 persons per year between 1976 and 1980 to 2.4/100,000 persons per year between 1991 and 1995 [11] followed by further growth of HCC incidence rate reaching 6.2/100,000 persons per year in 2011 as shown by SEER-based analysis [9].

Similarly, although death rates attributable to other frequent cancers, including lung, breast, colorectal and prostate cancers, are declining in USA, mortality from liver cancer has increased by 2.8% per year (2003–2012) in males and by 2.2% per year in females. The growing mortality from liver cancer in USA contrasts with the general decline in cancer mortality reaching 1.5% per year. Among all cancers, HCC is the fastest growing cause of death in the USA [2] and poses a significant economic burden on healthcare [10].

The spectrum of risk factors in HCC (see **Table 1**) explains the geographic heterogeneity. Awareness of these factors is important to understand the incidence and the associated needs for diagnostics and treatment. Worldwide, men have a higher incidence than women; gender ratio ranges around 3:1 both in global epidemiological studies of liver cancer [8] and more targeted analysis of HCC [9]. Incidence starts to increase at the sixth decade of life [2].

Liver cirrhosis of any aetiology represents the single largest risk factor of HCC and is found in 70–90% of cases. Worldwide, hepatitis B virus (HBV) infection accounts for more than 50% of HCC cases. In comparison to non-infected individuals, the relative risk of HCC is increased 100-fold in HBV-infected persons, and the risk further increases if HBV-infected patient develops cirrhosis, has longer duration of infection and higher virus burden in blood. The yearly risk of HCC in HBV-infected patients is 2% [18]. In East Asia and sub-Saharan Africa, HBV is the most common risk factor for HCC [12].

Hepatitis C virus (HCV) infection is implicated in 25–31% of patients [13, 19]. Although the presence of HCV infection holds 17-fold risk of HCC in comparison with non-infected persons [13], risk is significantly higher in cirrhotic patients. Thus, surveillance is limited to those having

Risk factor	Risk assessment	References
Hepatitis B virus infection	100-fold higher	[13]
Hepatitis C virus infection	17-fold higher	[13]
Alcohol consumption	2.2 times higher in people who consume at least 50 g of alcohol per day	[15]
Non-alcoholic fatty liver disease	More than 4 times higher	[16]
Aflatoxin exposure	Of the 550,000–600,000 new HCC cases worldwide each year, about 25,200–155,000 may be attributable to aflatoxin exposure	[14]
Primary biliary cirrhosis	Incidence of HCC is 3–5% per year	[12]
Primary sclerosing cholangitis (PSC)	The risk of HCC for PSC patients with cirrhosis is up to 2% per year	[17]
Hemochromatosis	Approximately 20-fold higher	[12]

Table 1. Risk factors of hepatocellular carcinoma [12–17].

HCV-associated cirrhosis or advanced fibrosis [12]. Annually, HCC develops in 2–8% of HCV-infected patients [13]. In North America, Latin America, Europe and Japan, HCV infection, together with alcohol abuse, represent the main risk factors [3, 13]. In Europe and Japan where HCV infection spread earlier than in the United States, HCC incidence has almost reached a plateau, while in the United States it is still increasing. HCV infection may have a synergistic effect with other risk factors, such as non-alcoholic fatty liver disease [3].

Globally, 15% of HCC cases can be attributed to alcohol-induced liver damage and non-alcoholic steatohepatitis [19], although the estimates range between 4 and 22% [20]. Non-alcoholic fatty liver disease (NAFLD) is the major hepatic manifestation of metabolic disturbances including obesity, type 2 diabetes mellitus, dyslipidaemia and metabolic syndrome [4]. As prevalence of these conditions is increasing, NAFLD has become the most common liver disorder in industrialised countries [21]. In NAFLD, HCC incidence reaches 44 (range, 29–66) per 100,000 person-years [22] contrasting with the general incidence of 6 per 100,000 in USA population [20]. The proportion of HCC related to NAFLD and non-alcoholic steatohepatitis (NASH) is increasing worldwide, especially in Western countries [20]. Although previously it was considered that HCC risk was limited to patients with liver cirrhosis, nowadays a significant fraction of NASH-associated HCC is found in non-cirrhotic liver or liver showing mild fibrosis [4].

Aflatoxins are a group of mycotoxins produced by *Aspergillus* fungi *(A. flavus; A. parasiticus)*, which can contaminate food products such as grains, rice, cassava, soybeans, corn and peanuts, stored in hot climate and high moisture. Aflatoxins are major risk factors of HCC in sub-Saharan Africa and Eastern Asia [23]. Chronic exposure to aflatoxin results in DNA damage, including mutation of the tumour suppressor gene *TP53* in hepatocytes [13]. In people subjected to aflatoxin ingestion and chronic HBV infection, HCC risk is 30- to 60-fold higher, *versus* HBV-uninfected people exposed to aflatoxin alone. Synergistic action is observed also between aflatoxin and HCV [14, 23, 24].

Planning the surveillance for individual patient, the presence of known risk factors must be considered and the relative risk must be taken into account. Organising surveillance measures in the society, population-attributable fraction (PAF) is also important. PAF depends both on relative risk and population prevalence of the corresponding risk factor. Thus, in USA, the risk increase of HCC is highest in HCV infection (odds ratio (OR), 39.9), followed by HBV infection (OR, 11.2), alcohol-induced liver disease (OR, 4.1) and diabetes mellitus and/or obesity (OR, 2.3). However, considering the prevalence of these conditions, diabetes and/or obesity are associated with the highest population attributable fraction (36.6%), followed by alcohol (23.5%), HCV (22.4%) and HBV (6.3%) as reported by Welzel et al. [25]. PAFs differ by the population. Worldwide, 54% of HCC occur in HBV-infected patients, 31% can be attributed to HCV and 15% to alcohol and NASH [19].

Considering the serious prognosis, early diagnosis is crucial, however, not always easy. Thus, correctly interpreted radiological findings, combined with biopsy when necessary, and appropriate immunohistochemical examination of biopsied tissues have diagnostic value. The molecular portrait of the tumour as well as easily available markers of the systemic inflammatory response, such as neutrophil-to-lymphocyte ratio or platelet-to-lymphocyte ratio, are recently reported to have prognostic and predictive value in HCC.

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The aim of the present chapter is to highlight the current approach and innovations for diagnostic evaluation of a liver nodule, suspected to be hepatocellular carcinoma. Non-invasive radiologic assessment represents the gold standard in certain patients. In contrast, difficult cases need biopsy evaluation, supplemented by immunohistochemistry, and may remain controversial even then.

2. Radiology

Radiological imaging and functional evaluation are significant in screening and diagnostics of HCC [26]. The gold standard techniques comprise ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI). A major advance in the diagnostics of HCC was reached in 2001, when non-invasive criteria were developed and accepted by the European Association for the Study of the Liver (EASL) to diagnose HCC in cirrhotic liver [27]. In addition to the presence of liver mass, radiologic studies of HCC evaluate the typical vascularity. HCC receives enhanced arterial blood supply reflected histologically by unpaired arteries. The blood supply via portal vein decreases in comparison with surrounding parenchyma. However, in early stages of development, HCC can be hypovascular if the portal flow has already decreased but the arterial supply has not yet fully developed.

According to the guidelines, ultrasonography is advocated for screening and surveillance of patients having high risk to develop HCC due to HBV or HCV infection, cirrhosis or other known risk factors [28]. The specificity is mostly higher than 90%, ranging 45–94% [5, 27]. The reported sensitivity ranges widely from 33 to 96%, at least partially because of differences in the equipment and qualification of radiologists [18]. The sensitivity decreases in advanced chronic liver disease because of the coarse cirrhotic nodularity seen both grossly and by US [5]. In a large group of 200 patients undergoing US and liver transplantation, the sensitivity for HCC diagnostics was 29.6% in regard to patients and only 20.5% counting the tumours themselves. Even a large tumour exceeding the diameter of 5 cm was missed [29].

The typical US presentation of HCC is a hypoechoic nodule although iso- or hyperechogenicity is possible as is nodule-in-nodule appearance [7]. Small HCCs (less than 2 cm in the greatest diameter) are mostly hypoechoic with or without posterior enhancement. Hyperechoic appearance is seen in 17% of small HCCs and can be associated with fat accumulation. Larger HCCs are heterogeneous reflecting necrosis (hypoechoic), calcifications and fibrosis. If hypoechoic halo (seen in 50% of HCC) and posterior enhancement is evident, these findings increase the specificity of diagnosis [5, 7, 18]. HCC in dysplastic nodule might seem hyperechoic within a larger hypoechoic area. If a nodule is identified on US, either CT or MRI is indicated for masses larger than 20 cm, while both methods are advocated for pathologic foci measuring 10–20 mm. If either CT or MRI confirms HCC, the diagnosis is reliable. Biopsy is indicated only for lesions that remain controversial after both imaging modalities. Nodules measuring less than 10 mm are followed up by US every 4 months [18].

By Doppler US, HCC is characterised by so-called basket pattern reflecting rich arterial vascularisation. Benign cirrhotic nodules feature either low vascularity or arterial vessels with

low frequency (high in HCC: >1 kHz) and normal resistive index (elevated in HCC: >0.71). However, the typical Doppler pattern is seen only in 50% of small HCC [7].

A significant innovation in ultrasonography is the application of contrast enhancement by stabilised gaseous microbubbles. Consequently, three phases can be assessed analogously to CT and MRI: arterial phase (beginning 20 seconds after injection and lasting for 30–45 seconds); portal venous phase (starting at 30–45 seconds and lasting for 2–3 minutes) and late phase (4–6 minutes). Some contrast agents display additional post-vascular phase characterised by contrast uptake in Kupffer cells (10–60 minutes). To avoid overlap with late phase, the post-vascular phase must be assessed not earlier than 10 minutes after contrast injection. The typical pattern of HCC upon contrast-enhanced ultrasound (CEUS) examination is arterial hyperenhancement followed by washout in the late phase. The evaluation of washout is important in order to exclude arterial hyperenhancement in hemangioma or dysplastic cirrhotic nodule. However, well-differentiated HCC can remain isoechoic in portal venous or late phase; such pattern is suspicious for HCC, but CT or MRI is mandatory [7]. The benefits of CEUS include the easy procedure and high safety as the technique is not associated with ionising radiation or renal toxicity. Pitfalls include false positives in cholangiocarcinoma [30]. The lack of specificity is associated with the intravascular location of microbubbles in contrast to CT or MRI contrast agents that reach the extravascular extracellular space. At present, CEUS has been excluded from diagnostic guidelines provided by the European Association for the Study of the Liver (EASL) and the American Association for the Study of the Liver Diseases (AASLD) but is advocated by Asian Pacific and Japanese guidelines [27].

US can be applied to recognise benign or secondary liver tumours. Sensitivity of US to detect liver metastases varies between 40 and 80%, again depending on experience of radiologist and available US equipment. Metastases can be hypovascular, e.g., gastric or colorectal carcinoma, or hypervascular as malignant melanoma or sarcoma [18].

If US or CEUS discloses a suspicious nodule, in-depth evaluation by CT or MRI is indicated [31] based on the risk of malignancy. Nodules smaller than 1 cm are mostly benign. Risk of HCC is 66% in nodules measuring 10–20 mm, 80% in nodules 20–30 mm in size and 92–95% in nodules larger than 3 cm [7]. Both methods (CT and MRI) are advocated for lesions measuring 10–20 mm while one is sufficient for larger nodules (>20 mm). The diagnosis of HCC is confirmed by the typical pattern of arterial hypervascularity and late washout [27].

For CT, dynamic multidetector row, multiphase contrast-enhanced computed tomography approach is recommended [27, 32] as the diagnosis is based on dynamic evaluation of blood flow. However, contrasting is not possible in all patients in order to avoid anaphylactic reactions, acute renal failure or hyperthyroidism [33]. To disclose HCC, CT must be evaluated in three phases (late arterial, portal venous and equilibrium) in addition to the first unenhanced image. HCC classically is hypervascular, characterised by high contrast in the arterial phase, followed by washout in portal and/or equilibrium phases [27]. Portal venous phase can be useful in some cases of HCC when the tumour is otherwise not visible in CT. Portal venous phase is generally less informative for HCC because the tumour shows rarefaction similar as liver parenchyma. This phase is most useful for detecting hypovascular metastases, e.g., from colorectal carcinoma [34].

Examination of hypovascular or hypervascular liver metastases with multidetector CT is similar to CEUS. Hypovascular metastasis presents as rounded and uniformly hypoattenuating mass in portal venous phase and peripheral rim in arterial phase. Hypervascular metastasis is characterised by homogeneous late arterial enhancement. Inhomogeneous enhancement can develop in necrosis or haemorrhage [35].

MRI has excellent results for detection and characteristics of HCC. By meta-analysis, MRI was characterised by sensitivity of 88% and specificity of 94%, exceeding the characteristics of multidetector CT [36]. Contrasting usually is applied in liver MRI, most frequently by gadolinium compounds. The gadolinium-based contrast agents can be classified as extracellular *versus* hepatobiliary. Extracellular agents are small molecules that can reach interstitium moving out from vascular space. In turn, hepatobiliary contrast agents move even further becoming absorbed by hepatocytes [37, 38].

Classic MRI protocol for HCC includes a 3D T1-weighed fat saturated sequence with intravenous contrast. The first phase is called late arterial phase. It is seen 25–30 seconds after injection of contrast. This phase is followed by portal venous phase, at 65–70 seconds. In this phase, there is a dense contrast enhancement in portal vein, and hepatic veins also become highlighted. Finally, delayed phase develops 3 minutes after injection [38]. Before contrasting, classical HCC is hypointense in T1-weighted and hyperintense in T2-weighted images. Contrasting reveals similar enhancement pattern as in CT with arterial enhancement and subsequent washout [18]. In addition, MRI can be applied to disclose tumour thrombus in portal venous system [39].

Most metastases show mild-to moderate high signal intensity on T2-WI. In some cases, e.g., in cystic or necrotic metastases, T2 signal increases.

The sensitivity of MRI can be further improved by diffusion-weighted imaging, based on the assessment of Brownian motion of water molecules and water diffusion within a voxel (a tridimensional pixel). Cell membranes limit the diffusion, therefore greater cellularity, seen also in malignant tumours, results in diffusion restriction [40]. However, the fibrosis also decreases the mobility of water molecules. By different modalities, diffusion-weighted imaging can increase the sensitivity for HCC detection, the liver-to-lesion contrast and the specificity in the differential diagnosis with benign cirrhotic nodules [27].

Another advance in liver pathology is represented by hepatobiliary phase MRI using contrast agents that are absorbed by hepatocytes and excreted in biliary system, e.g., gadoxetate disodium and gadobenate dimeglumine. These agents undergo dual elimination via biliary excretion (50%) and renal glomerular filtration, while the traditional agents, as gadopentetic acid, are almost completely excreted via kidneys [41]. The hepatobiliary phase of MRI corresponds to the peak parenchymal enhancement due to contrast uptake in hepatocytes. Depending on the agent, the hepatobiliary phase develops either 10–20 (gadoxetate) or 60 (gadobenate dimeglumine) minutes after injection [42]. Most of HCCs are hypointense in hepatobiliary phase [18].

MRI can be applied to distinguish between HCC and benign lesion in non-cirrhotic liver. In such patients, HCCs are hypointense in T1, hypo- or hyperintense in T2, lack central

enhancement in the tumour, exhibit satellite lesions and do not uptake liver-specific contrast agents [43].

Positron emission tomography (PET) is a non-invasive radiologic visualisation that demonstrates metabolic activity in normal or pathological tissue. It is usually performed in combination with CT to ensure both anatomical imaging and metabolic evaluation. 18-fluorodeoxyglucose (FDG) is one of the radiopharmaceuticals used in PET/CT. It discloses areas of high glucose uptake as many tumours including HCC are characterised by aerobic glycolysis: the Warburg effect [44].

The significance of FDG PET/CT in HCC evaluation is not unequivocal. The distinction between small, well-differentiated HCC *versus* regenerative or dysplastic nodules can be difficult. The positive aspect of PET/CT is the ability to detect extrahepatic metastases of HCC. Considering that PET/CT provides whole-body examination, it is recommended before liver transplantation [45, 46]. Hypothetically, prognostic role of PET/CT in HCC has been discussed as well as the ability to predict response to treatment [46]. Other radiopharmaceuticals are also under discussion, including lipid radiotracer on choline base, like 11C-choline or 18F-fluorocholine [47]. 68Ga-labelled prostate-specific membrane antigen, that is used to diagnose prostate cancer, is present in other tumours, including HCC [48].

3. Pathology

Needle biopsies followed by morphologic and immunohistochemical examination can be invaluable for the characterisation of liver masses. However, nowadays clear-cut radiologic diagnostic criteria have been established for the non-invasive diagnostics of HCC; therefore, the advantages and indications of the biopsy should be considered against the risks and contraindications. Liver biopsy is recommended only in selected patients, thoughtfully evaluating the diagnostic yield [6].

Currently, three general groups of indications (see **Table 2**) for liver biopsy are known: to establish the diagnosis, to assess the prognosis and/or to assist in the management of patient

Diagnosis:
• Identification and staging of parenchymal and cholestatic liver diseases (alcohol-induced liver disease; non-alcoholic steatohepatitis; primary biliary cirrhosis; primary sclerosing cholangitis; Wilson's disease, haemochromatosis)
• Evaluation of persistent abnormal liver biochemical tests
• Evaluation of the type and extent of drug-induced liver injury
• Diagnosis of multisystem infiltrative disorders
• Identification and determination of the nature of focal/diffuse intrahepatic abnormalities on imaging studies
Prognosis and management:
• Pre-treatment evaluation and staging of chronic hepatitis, e.g. chronic viral hepatitis B and C
• Evaluation of pre-transplant living-related donor
• Evaluation of post-transplant patient with abnormal liver tests (rejection *versus* infection)
• Evaluation of treatment efficacy for liver diseases

Table 2. Indications for liver biopsy [49].

with known liver disease [49]. Percutaneous liver core biopsy is most frequently performed to evaluate the presence and activity of inflammation and extent of fibrosis/stage of frequent liver diseases, mostly chronic viral hepatitis, alcohol-induced liver disease and NAFLD. Regarding focal liver lesions, biopsy can yield the diagnosis. Molecular analyses of tissue may help determine the most appropriate individual treatment strategy for the patient with HCC [50] but are still under development for HCC. At present, biopsy from a nodule in cirrhotic liver is indicated if the findings of radiological imaging are controversial [6].

Although biopsy is often essential, sometimes it may be difficult to undertake because of associated risks (see **Table 3**). Percutaneous, ultrasound-guided liver biopsy (the Menghini method) has become the worldwide standard [51]. However, it is appropriate only in cooperative patients. Thus, if the patient refuses from the procedure, it is absolutely contraindicated. Although precise blood clotting parameters are unsettled, coagulopathies should be mentioned as a serious contraindication [49]. In this case, mini-laparoscopy or transjugular liver biopsy might be considered [51]. Among relative contraindications, ascites should be pointed out, as it may prevent adequate sampling of tissue, as well as increase the risk of bleeding [49]. Biopsies of malignant liver lesions also carry a low risk of tumour seeding.

Significant complications due to liver biopsy arise in about 1% of cases, with less than 0.1% mortality [51]. The main complications are post-interventional haemorrhage and bile leakage; others, like injuries to gall bladder, lung, kidney, as well as bacteraemia are rare [49, 51].

The initial assessment of liver tissue starts with the overall evaluation of parenchymal architecture. Haematoxylin and eosin represents the generally accepted standard stain in liver pathology [6]. Helpful additional visualisation methods in liver pathology include Masson's trichrome to assess fibrosis, Gordon and Sweets reticulin to evaluate lobular architecture and hepatocyte plate thickness, Perl's iron to detect hemosiderin deposits and periodic acid-Schiff (PAS) stain to identify glycogen, mucus or chitin of certain liver parasites.

Microscopically, cells of classical HCC resemble normal hepatocytes. The similarity to normal liver is most notable in well to moderately differentiated tumours. In such cases, the loss of the normal liver cell plates and plate thickness change from 1 to 2 cell nuclei to 3 or more nuclei

Absolute contraindications
• Uncooperative patient
• History of unexplained bleeding
• Tendency to bleed (prothrombin time more than 3–4 seconds over control; platelet count <50,000 mm^3; prolonged bleeding time (≥10 minutes))
• Unavailability of blood transfusion support
• Recurrent use of aspirin or other non-steroidal anti-inflammatory drugs within last 7–10 days
Relative contraindications
• Ascites
• Morbid obesity
• Infection in the right pleural cavity or below the right hemidiaphragm
• Suspected haemangioma or other vascular tumour
• Suspected hydatid disease (Echinococcal cysts)

Table 3. Contraindications of liver biopsy [49].

across a single neoplastic cord is a feature of malignancy. In healthy liver, narrow cords of hepatocytes are running in parallel, but even well-differentiated HCC shows a disorganised pattern secondary to the increased thickness of the hepatocyte cords (usually more than 3 cells thick), that can be highlighted by reticulin staining. The invasive growth of HCC disrupts and destroys the liver plate architecture, leading to decreased amount of reticulin and disorganised pattern of it. However, the loss of reticulin is not complete. HCC is characterised by the absence of normal portal tracts and/or naked or unaccompanied arteries in accordance with the radiologic hypervascularity and high contrast in the arterial phase of contrast-enhanced CT [6]. Invasion in connective tissues is diagnostic. However, except scirrhous and fibrolamellar HCC, stroma is usually scant in HCC. Loss of perinodular ductular proliferation is a manifestation of invasive growth [6]. Vascular invasion is diagnostic if evident.

Cytologically, HCC shows both signs of hepatocellular differentiation that serves as the clue to hepatocellular origin of the tumour and atypia indicating malignant behaviour. Regarding tumour differentiation, bile production is a reliable indicator of hepatocellular origin. Bile can be found in the cytoplasm of neoplastic cells or in lumina of acinar complexes. Similarly to benign counterparts, steatosis, Mallory bodies and hyaline globules can develop in cytoplasm of tumour cells. HCC cells can have intranuclear inclusions and/or optically clear cytoplasm. Giant cells are occasionally present. Iron accumulation in cells of hepatocellular carcinoma is not seen, even in the setting of hereditary hemochromatosis. In hepatocytes, nuclear pleomorphism can be a feature of regenerative changes; therefore, mitotic activity is more suspicious of malignancy, and the presence of atypical mitoses definitively confirms the presence of a malignant tumour. However, in well-differentiated HCC, abnormal mitoses are rare and are not mandatory for diagnosis [6].

The histologic patterns of HCC include trabecular (the most common pattern), acinar (pseudoglandular), solid and scirrhous patterns. Trabecular HCC resembles normal liver architecture. In acinar or pseudoglandular HCC, the neoplastic cells are arranged in gland-like tubules containing bile or fibrin. Solid HCC is characterised by compact, sheet-like arrangement of neoplastic cells. Scirrhous HCC exhibit marked desmoplasia; it will be described in detail later. HCC is characterised by significant inter- and intratumoural heterogeneity, manifesting as variability of grade and growth patterns [3]. Grade progression can be present even in a single patient and, in fact, reflects the biology of HCC. Hepatocellular carcinoma frequently develops in foci with equivocal biological potential, e.g., dysplastic cirrhotic nodule. Such early HCC typically is well differentiated. Over the disease course, well-differentiated HCC progresses to advanced dedifferentiated tumour. The heterogeneity can lead to diagnostic problems and failures in biopsy due to sampling errors. For instance, if a small suspicious nodule was evident by radiological imaging and a biopsy was obtained, the differential diagnosis between dysplastic nodule and HCC will frequently imply the necessity to distinguish between premalignant process and well-differentiated tumour, usually lacking marked cell atypia or clear-cut invasion. In addition, both processes can be adjacent in the tissues.

HCC has several histologic variants, including fibrolamellar, sarcomatoid, scirrhous, steatohepatitic and clear cell HCC, presenting with peculiar morphological features. Some cases display lymphoepithelioma-like morphology. In addition, correlations between histological and molecular subtypes have been reported [52].

Fibrolamellar HCC is a rare subtype accounting for less than 1% of HCC. Typically, fibrolamellar carcinoma is diagnosed in young adults lacking liver cirrhosis or other known predisposing factors [3]. The mean age of diagnosis is 26 years [53]. Association with germline pathogenic variants of *TP53* gene has been reported suggesting that some cases of fibrolamellar HCC might represent Li-Fraumeni syndrome. Interestingly, in the case described by Andrade et al., a germline mutation of *TP53* was identified not only in proband affected by fibrolamellar HCC but also in her asymptomatic mother [54].

The presence of fibrous septae and central scar with possible calcification leads to architectural similarity with focal nodular hyperplasia [3, 6]. Histologically, the neoplastic cells are arranged in trabeculae and sheets, separated by collagen fibres that undergo hyalinisation and show the typical lamellar pattern [3]. Fibrolamellar HCC is defined by triad of histologic features: (1) large, polygonal neoplastic hepatocytes with wide eosinophilic granular cytoplasm. Ground glass pale bodies and PAS-positive cytoplasmic globules can be present [3, 53] but are neither sufficient nor necessary for diagnosis. (2) Prominent single eosinophilic macronucleoli should be present, and frequently are seen on the background of vesicular chromatin structure [3, 6]. (3) Lamellar fibrosis, usually present in at least half of the tumour tissue [53].

The immunophenotype of fibrolamellar HCC is also unusual, showing expression of hepatocellular markers in combination with biliary, progenitor and stem cell features as well as macrophage markers (CD68). The granular or dot-like expression of CD68 in association with appropriate morphology is helpful in diagnosing fibrolamellar HCC [6].

Prognosis of fibrolamellar HCC is poor. The 5-year survival is similar to conventional HCC arising in non-cirrhotic liver [53]; however, it is better than for classical HCC arising in cirrhotic liver [3].

Sarcomatoid HCC can occur either primarily or within classical HCC [3]. This subtype, comprising 1.8–3.9% of HCC, is partially or fully composed of malignant spindle-shaped cells, occasionally showing heterologous (rhabdoid, osteoid or chondroid) differentiation [53]. If there is no adjacent area of classical HCC, it is difficult to distinguish sarcomatous HCC from true sarcomas, including primary or metastatic tumours, e.g., metastatic gastrointestinal stromal tumour, leiomyosarcoma or fibrosarcoma. Haematoxylin-eosin stain alone can be insufficient, necessitating immunohistochemistry [3]. Considering the high grade and remarkable anaplasia of sarcomatoid HCC, hepatocellular markers should be supplemented with pancytokeratin and specific markers for sarcoma, including CD117, DOG, actin, desmin, S-100, CD34 and CD31. Hepatocellular antigens are frequently negative, and even pancytokeratin is expressed only in 23–63% cases of sarcomatoid HCC [53]; therefore, complex assessment of morphology is mandatory along with clinical history and IHC for sarcoma.

Scirrhous HCC is a rare type, accounting for 0.2% to 4.6% of HCC. It can develop beneath liver capsule leading to pedunculated gross view [3, 53]. Microscopically, scirrhous HCC is characterised by diffuse fibrosis surrounding thin trabeculae of neoplastic cells. Such fibrosis can occur either after various regimens of oncologic treatment (chemotherapy, transarterial chemoembolization, irradiation) or in untreated patients [3]. However, HCC exhibiting post-treatment fibrosis should not be classified as scirrhous [53]. The marked desmoplasia and morphology of the tumour cells, displaying clusters, strands and tubules, can lead to misdiagnosis as cholangiocarcinoma or

metastasis both in biopsy and in preoperative imaging. While conventional HCC is characterised by CT enhancement in the arterial phase and washout in the venous phase, scirrhous HCC can present with peripheral ring-like enhancement in the arterial phase and delayed central enhancement in the venous phase [53]. In addition, expression of cytokeratin 19 is frequent [52]. Haemorrhage or necrosis is usually absent. Marked CD8-positive lymphocytic infiltrate can be present [3, 53]. Regarding molecular profile, scirrhous HCC is associated with mutations in *TSC1*/*TSC2* genes, lack of *CTNNB1* mutations, presence of epithelial to mesenchymal transformation and stem cell profile [52].

Lymphoepithelioma-like carcinoma is characterised by the presence of rich lymphocytic infiltrate surrounding pleomorphic, small, polygonal neoplastic cells that might show syncytial growth [1].

Steatohepatitic HCC is remarkable for similarity to steatohepatitis that can even lead to missed diagnosis in well-differentiated cases [53]. This subtype HCC is characterised by the presence of fat vacuoles in more than 5% of the tumour. The neoplastic cells also show Mallory bodies and ballooning degeneration. The stroma features pericellular and trabecular fibrosis as well as inflammatory infiltrate, consisting of neutrophils, plasma cells and lymphocytes [3]. Infiltrative borders are characteristic. Within the tumour, fibrosis can be prominent [53]. The patients can have underlying steatohepatitis due to metabolic syndrome/NASH [3] or alcohol-induced liver disease [53]. However, this phenotype of carcinoma is also seen in patients without steatohepatitic changes in the non-neoplastic liver tissue [3]. Molecularly, IL6/JAK/STAT molecular pathway is frequently activated along with immunohistochemical C-reactive protein expression. In contrast, mutations in *CTNNB1* gene or activation of Wnt/Beta-catenin pathway are not evident. Regarding immunophenotype, low expression of glutamine synthetase has been observed [52].

Clear cell HCC features optically clear cytoplasm due to the presence of glycogen and fat vesicles in the neoplastic cells. The architecture is mostly trabecular [3].

The differential diagnosis of HCC includes benign pathological processes, for instance, dysplastic nodule in a cirrhotic liver while hepatic adenoma, focal nodular hyperplasia and bile duct adenoma should be considered in non-cirrhotic liver. Parasitic infestations, e.g., echinococcosis and infrequent benign tumours, e.g., angiomyolipoma occasionally need to be ruled out. The malignant tumours that enter the spectrum of differential diagnoses of hepatocellular carcinoma include metastases of extrahepatic tumours as well as cholangiocarcinoma, hepatoblastoma and non-epithelial liver tumours.

3.1. Immunohistochemistry and differential diagnosis

Benign and malignant liver tumours may share morphologic similarities; thus, immunohistochemical assessment is crucial to set the correct diagnosis. The two challenging tasks are (1) to distinguish low-grade/early HCC from benign lesions like liver adenoma, focal nodular hyperplasia or dysplastic nodule and (2) to differentiate high-grade HCC from metastatic tumours in the liver.

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The differential diagnosis of HCC varies also depending on the underlying liver pathology. In cirrhotic liver, primary tumours such as HCC and cholangiocarcinoma are much more common than secondary tumours [3]. In contrast, in non-cirrhotic liver, HCC accounts only for about 2% of tumours and metastatic lesions predominate over primary liver neoplasms. Metastasis can mimic HCC, especially in case of clear cell renal cancer, clear cell adenocarcinoma of the female genital organs, hepatoid gastric carcinoma, adrenal carcinoma and melanoma. Metastatic gastrointestinal neuroendocrine tumours can be challenging to differentiate from HCC, especially if trabecular architecture is present [3]. In the evaluation of HCC diagnosis, arginase-1, hepatocyte paraffin-1 antigen, glypican-3, carcinoembryonic antigen by polyclonal primary antibody, CD10, glutamine synthetase and CD34 are frequently assessed. Alfa-fetoprotein is partially replaced by new markers showing higher expression frequency and less background. However, it is still helpful in some cases. Clathrin and bile salt export pump protein represent promising novel targets.

Arginase-1 (Arg1) is occasionally considered the most sensitive and specific marker of hepatocellular differentiation [55], characterised by sensitivity and specificity of approximately 90% [55]. Arginase-1 represents manganese metalloenzyme involved in the urea cycle [56]. It catalyses the hydrolysis of arginine to ornithine and urea. Arg1 is expressed in normal human liver [6] and hepatocellular tumours, including HCC. Arg1 shows better sensitivity and specificity diagnosing HCC, compared to HepPar1 and glypican 3 [55], although other researchers prefer HepPar1 (see further) to identify hepatocellular differentiation [3]. Regarding the types of HCC that might cause diagnostic difficulties—high-grade HCC and scirrhous HCC—Arg1 is characterised by sensitivity of 85 and 85%, exceeding the sensitivity of HepPar1 (64 and 26%, respectively). Arg1 displays diffuse nuclear and cytoplasmic expression pattern in HCC [6, 55]. Most other tumours are negative for Arg1, but focal or weak expression can occur in colorectal, pancreatic, breast and prostatic carcinomas, cholangiocarcinoma or hepatoid tumours [55].

Hepatocyte paraffin-1 (HepPar1) antigen is another marker of hepatocellular differentiation. Some authors prefer HepPar1 as the best marker to confirm the hepatocellular origin of a tumour [3]. HepPar1 is a carbamoyl phosphate synthetase 1: another enzyme involved in urea synthesis. In contrast to Arg1, it is expressed not only in the liver but also in non-neoplastic small intestinal mucosa and Barrett's oesophagus [56]. HepPar1 has diffuse granular cytoplasmic staining pattern. The sensitivity and specificity in HCC reaches 80%. HepPar1 is expressed in almost all well-differentiated HCCs. However, only less than 50% of high-grade cases express HepPar1 [3]. Most of metastatic and/or non-hepatocellular tumours, including adenocarcinomas, neuroendocrine tumours, renal cell carcinoma, adrenocortical carcinoma, melanoma and angiomyolipoma, are negative for HepPar1. However, focal reactivity is occasionally observed. Strong expression can be present in cholangiocarcinomas and metastatic oesophageal, gastric and pulmonary adenocarcinomas [55]. Positive reaction has also been reported in non-ampullary small intestinal adenocarcinomas (60%) and ampullary adenocarcinomas with intestinal (73%) differentiation while expression in ampullary adenocarcinomas exhibiting pancreatobiliary (14%) morphology or colonic (9%) adenocarcinomas is rare [56].

Glypican-3 (GPC3) is a member of the glypican family of heparan sulphate proteoglycans. It is bound to the external surface of plasma membrane through a glycosyl-phosphatidyl-inositol

anchor. Glypicans regulate signalling via Wnt, Hedgehog, fibroblast growth factor and bone morphogenetic protein pathways. Thus, glypicans are involved in the control of cell proliferation. In HCC, GPC3 promotes cancer growth by stimulating Wnt signalling. The GPC3 molecule can be released to extracellular environment after it has been cleaved off by lipase [57]. Hence, the functional activity of GPC3 explains its role as possible serum marker or treatment target for HCC. GPC3 is normally found in foetal liver and placenta but is absent from healthy adult liver and benign hepatocellular lesions including focal nodular hyperplasia and liver adenoma [55]. Thus, expression of GPC3 in liver biopsy is highly suggestive of HCC. The staining pattern is (1) granular or diffuse cytoplasmic, possibly with membranous enhancement; (2) membranous or (3) Golgi complex-related [6, 55]. Heterogeneity can lead to focal lack of expression; therefore, negative result in biopsy does not exclude HCC. The sensitivity of GPC3 ranges from 56 to 62% in low grade (G1) HCC to 80–83% in intermediate grade (G2) HCC, 85–89% in high grade (G3) HCC and 79% in scirrhous HCC [55]. GPC3 is expressed in many extrahepatic tumours that can spread to the liver, including metastatic adenocarcinoma, squamous cell carcinoma, non-seminomatous germ cell tumours (choriocarcinoma, yolk sac tumour) and malignant melanoma (5%). Cholangiocarcinoma can be positive (5%) as well [6, 55]. The strong advantages of GPC3 include the absence of it from non-malignant liver as well as high sensitivity in high-grade HCC. Lack of specificity is the greatest pitfall [55].

Carcinoembryonic antigen (CEA) family represents a class of different glycoproteins belonging to immunoglobulin superfamily. Within CEA family, adhesion molecules and pregnancy-specific glycoproteins are distinguished. The functions of CEA family include cell adhesion, as well as cell interaction in pregnancy, immune reactions and angiogenesis [58]. By immunohistochemistry, CEA is found in foetal and adult epithelial cells [6]. In liver pathology, CEA assessment by polyclonal antibody (pCEA) is strongly advised. In HCC, distinct specific canalicular or so called chicken-wire fence pattern can be observed. Metastatic adenocarcinomas show diffuse membranous, luminal and/or cytoplasmic positivity [55]. In higher grade HCC, the specific canalicular pattern is progressively lost and replaced by unspecific membranous expression [6].

CD10 is a zinc-dependent metalloproteinase, located in cell surface membranes. It exhibits neutral endopeptidase activity: cleavage of peptides at the amino side of hydrophobic residues. CD10 inactivates several hormones, as glucagon, oxytocin and bradykinin. In HCC, CD10 shows canalicular expression similarly to pCEA. However, the sensitivity of CD10 for HCC is lower, around 50% [55].

Alpha-fetoprotein (AFP), the protein encoded by *AFP* gene on 4q25, is the major plasma protein in developing foetus. It is produced by liver and yolk sac and might represent the foetal analogue of albumin. AFP can bind metal ions, fats and bilirubin. In adults, AFP is found in HCC and germ cell tumours but normal liver tissue does not express AFP [3]. Although the sensitivity of AFP for HCC is only 30–50% and high background can frequently limit the interpretation [55], truly positive cases in our experience were easy to recognise. In contrast to HepPar1 and pCEA, AFP positivity increases with dedifferentiation of HCC [3].

Glutamine synthetase (GS) is an enzyme that catalyses the condensation reaction between glutamate and ammonia resulting in glutamine. GS is regulated by beta-catenin molecular pathway. In normal liver tissue, immunohistochemical expression of glutamine synthetase is

found only in a thin central perivenular (zone 3) area. In contrast, extensive diffuse cytoplasmic expression is present in 70% of HCC [6].

CD34 has multiple diagnostic roles. Within its wide expression spectrum, endothelial cells are also positive. Sinusoidal expression of CD34 is increased in both benign and malignant hepatocellular lesions, contrasting with limited expression in periportal sinusoids within normal liver [55] or in parenchymal capillaries close to fibrous septa within cirrhotic tissues [6]. In HCC, the endothelial expression of CD34 increases, until capillarisation of the sinusoids becomes complete. The capillarisation develops due to higher oxygen tension in HCC. Although incomplete CD34 expression does not exclude HCC, diffuse positive reaction is strongly suggestive of HCC. However, limited sampling in biopsy can lead to pitfalls as foci of complete CD34 expression are seen in adenomas and in periphery of cirrhotic nodules. If such foci are predominantly sampled within the biopsy, false overestimation of CD34 reactivity is possible [6].

Clathrin is one of the novel markers appearing in the differential diagnostics between malignant and non-malignant hepatocellular nodules. Clathrin is a protein that forms airscrew-like triskelion consisting of three light chains and three heavy chains. When these molecules assemble between themselves, clathrin-coated vesicles arise and participate in endocytosis and exocytosis. Thus, clathrin participates in cell communication and signalling, in the transport of nutrients, receptors and other macromolecules. During mitosis, clathrin stabilises mitotic spindle. The heavy chain of clathrin is significantly upregulated in HCC. In the initial reports, striking contrast in the immunohistochemical staining was found between tumour and surrounding tissues suggesting high affinity and low background. The expression pattern was cytoplasmic and membranous. Expression of the heavy chain of clathrin was tested for the distinction between HCC and benign nodules. The sensitivity and specificity of the heavy chain of clathrin was 41.2 and 77.2%, and the sensitivity increased to 61.1% in combination with glypican-3 [59].

Bile salt export pump protein is a transport molecule that is present in bile canaliculi. By immunohistochemistry, bile salt export pump protein was expressed in 89.6% HCC, mostly (76.7%) in canalicular pattern. In comparison with cholangiocarcinomas and metastatic tumours, expression of bile salt export pump protein had 90% sensitivity and 100% specificity for HCC. The performance of bile salt export pump protein was comparable to arginase-1 showing both sensitivity and specificity of 94% and slightly better than HepPar1 characterised by sensitivity 90% and specificity 97% [60].

3.2. Well-differentiated hepatocellular carcinoma *versus* adenoma

Hepatocellular adenoma (HCA) is defined as benign monoclonal proliferation of well-differentiated hepatocytes. The most common risk factor for HCA is exposure to high oestrogen levels in oral contraceptives, thus the disease has strong female predominance (9:1). Adenomas are typically small, solitary lesions in non-cirrhotic liver. Occasionally, multiple tumours are observed [61]. In HCA, the neoplastic hepatocytes are arranged in cords and sheets, typically two layers thick [3, 62]. The portal triads and interlobular bile ducts are absent from adenoma tissue [63]. Pseudoglandular architecture can be observed, especially in adenomas associated

with anabolic use. HCA cells appear larger due to intracellular glycogen or fat accumulation. Nuclear atypia is absent [3].

Several molecular subtypes of hepatocellular adenomas are known [62, 64], including hepatocyte nuclear factor 1α (HNF1α) inactivated type (H-HCA); β-catenin activated type (B-HCA); inflammatory HCA (I-HCA) and the unclassifiable type (U-HCA). Not surprisingly, beta-catenin activated subtype is associated with malignant transformation [62]. Beta-catenin mutations are reported in 20% of HCCs, especially in patients with underlying hepatitis C virus infection. HCC arising from B-HCA is usually well to moderately differentiated and lacks vascular invasion or satellite nodules [3]. Mutations lead to remarkable overexpression of *GLUL* gene (coding for glutamine synthase), thus beta-catenin activation can be assessed by intense homogeneous cytoplasmic expression of glutamine synthase and by aberrant nuclear localisation of beta-catenin [62, 63]. H-HCA shows decreased expression of liver fatty acid-binding protein, and presence of fat in neoplastic cells can be seen histologically. I-HCA is characterised by immunohistochemical positivity for serum amyloid A and C-reactive protein. Marked inflammatory infiltrate, ductular reactions and sinusoid dilation can be present in the tissue as well. U-HCA lacks gene mutations or specific immunohistochemical findings, but is diagnosed as HCA by histology [61]. Liver adenomas express hepatocellular markers and have lower proliferation activity than HCC [63]. To discriminate between adenoma and HCC, the following parameters are of importance: (1) clinical history in order to disclose risk factors that might indicate either HCA or HCC; (2) structure of surrounding liver as presence of cirrhosis favours HCC; (3) expression of HCA subtype-specific proteins; (4) presence or absence of cell atypia and invasion; (5) hepatocyte plate thickness and (6) expression of malignancy-associated HCC markers, e.g., GPC3.

3.3. Well-differentiated hepatocellular carcinoma *versus* focal nodular hyperplasia

Focal nodular hyperplasia (FNH) is a hyperplastic hepatocellular proliferation resulting from blood flow abnormalities. It is a pathological focus characterised by nodular architecture, hypervascular central scar associated with thick fibrous septa between hepatocyte nodules, inflammatory infiltrate, presence of ductular reaction and sinusoid dilation [55, 61–63].

To distinguish FNH from HCC, GPC3, heat shock protein 70 (HSP70) and reticulin network can be assessed. Loss of reticulin framework, immunohistochemical expression of GPC3 and/or diffuse nuclear expression of HSP70 favours HCC. Such immunohistochemical evaluation has 100% specificity for HCC although the sensitivity is only 43–46%. Typical "map-like" pattern of GS expression is evident in FNH. It is characterised by wide central positive areas in the middle of nodules. The positive foci interconnect between themselves, while periseptal areas remain negative. This reactivity pattern contrasts with normal liver showing limited perivenular reactivity in the middle of lobules [55].

3.4. Well-differentiated hepatocellular carcinoma *versus* high-grade dysplastic cirrhotic nodule

Dysplastic cirrhotic nodules (DNs) are characteristic precursors of HCC in the setting of chronic liver disease and/or liver cirrhosis. Most but not all dysplastic nodules are small, not

exceeding the diameter of 1 cm [6]. Morphologically DNs are classified into high-grade DN and low-grade DN. Low-grade DN, carrying low risk of transformation to HCC, is generally characterised by monotonous cell population when compared with the surrounding cirrhotic liver, mildly increased cell density and minimal cell atypia. The nuclear/cytoplasmic ratio is mildly increased, nuclear atypia is slight, mitoses are absent and cell plates are 1–2 cells thick. The reticulin network is retained. The borders of low-grade dysplastic nodule are rounded, but the adjacent liver parenchyma is not compressed [3]. In contrast, high-grade dysplastic nodules can have many of classical HCC features. The nuclear/cytoplasmic ratio is increased. Nuclei show hyperchromasia and irregular borders and can be peripherally located. Occasional mitoses can be present. Cell plates are thicker than 2 cells. Cytoplasm switches to basophilic staining. Pseudoglandular structures start to appear. Occasional unpaired arteries have been observed. Lack of invasion is the most reliable criterion in the differential diagnosis with early HCC [3]. This trait is both important and biologically substantiated as the invasion is the hallmark of malignant tumours. However, it can be notoriously difficult to apply practically. In early HCC, invasion can be absent from biopsy due to sampling error. Regarding high-grade dysplastic nodule, entrapment of perinodular hepatocytes into fibrous tissues mimics invasion. To classify the entrapped hepatocytes correctly, immunohistochemical investigation of ductular proliferation can be helpful, as further described, because these non-neoplastic intraseptal hepatocytes and ductular proliferation stem from common progenitors [3].

Expression of GPC3 points towards malignant hepatocellular tumour, as it was previously noted. However, GPC3 expression has been reported in 3–76% of dysplastic nodules. Glutamine synthetase is expressed in 69.8% of HCC contrasting with 13.6% in high-grade DN. Heat shock protein 70 is found in 73.5% of HCC and only exceptional dysplastic nodules [3]. To distinguish high-grade DN from early HCC, immunohistochemical panel comprising heat shock protein 70, glypican-3 and glutamine synthetase has been recommended. Expression of one marker is compatible with DN, while HCC expresses at least two markers. The sensitivity of this panel is estimated as 60–78% [55].

In addition, cytokeratin (CK) 7 and/or CK19 and CD34 can be useful in the assessment of architecture and reactive changes. HCC is characterised by more diffuse expression of CD34 and loss of ductular reaction at the nodule interface. Dysplastic nodule shows only focal CD34 expression in the periphery of the nodule and more marked proliferation of CK7-positive ductules surrounding DN [55]. In the ductular reaction, CK7 and CK19 usually are coexpressed. Thus, gradual loss of CK7 and CK19 positive ductular reaction in perinodular stroma correlates with progression of cirrhotic to dysplastic nodule and further to HCC. Ductular reaction is present around ≥50% of perimeter of a DN, while it is almost lost in HCC [3].

Different systems for complex evaluation of the biological potential of hepatocellular nodule have been proposed. Integrated evaluation of haematoxylin-eosin findings together with reticulin stain and immunohistochemistry for CD34 has been suggested. A hepatocellular nodule should be classified as HCC if at least three features from the following are present: necrosis; cellular atypia; thickness of trabeculae more than 4 cells; mitotic activity or diffuse expression of CD34 in the sinusoidal endothelium [6]. Alternatively, stromal invasion, loss of reticulin network and positivity for at least two out of three markers (HSP70, GS, GPC3)

are considered the strongest parameters discriminating HCC from high-grade dysplastic nodule [3].

3.5. Hepatocellular carcinoma *versus* metastasis

If high-grade malignant tumour is found in the liver, the differential diagnosis includes metastatic malignancy *versus* HCC and cholangiocarcinoma. Any malignant tumour can ultimately spread to the liver via bloodstream, lymphogeneous dissemination or transperitoneal spread. In some biopsy series, metastatic lung, colorectal, pancreatic and breast carcinomas have been the most common secondary liver tumours [3]. However, frequency of different metastatic malignant tumours in liver biopsies depends on many factors, including the biological potential of the tumour and its incidence in the population as well as institutional approach to liver biopsy in different oncological patients. This, in turn, may depend on the patient's general status, presence of contraindications for biopsy or significant oncological treatment and the availability of effective treatment.

In order to distinguish HCC from metastatic tumours, it is advisable to combine at least two hepatocellular markers and at least two antigens that are more frequently seen in adenocarcinomas. Among hepatocellular markers, Arg1 should be combined with either HepPar1 or GPC3. Most of adenocarcinomas express cytokeratin (CK) 19, MOC-31 and CK7 [55]. The spectrum of immunohistochemical panel should be planned in accordance with tissue availability within the biopsy. The suggested minimal panel includes ARG1 and CK19 [55], while maximal investigation might include several HCC markers accounting for different grades of HCC, several adenocarcinoma markers and antigens that are characteristic for certain tissues (neuroendocrine or melanocytic differentiation) or epithelia of specific organs, e.g., breast, large bowel, lung, thyroid, kidney and others. Panels of immunohistochemical markers can disclose the location of primary tumour giving rise to metastasis. Thus, CK20 and CXD2 are typical for metastatic colorectal carcinoma; CDX2 and CK7 for gastric carcinoma; TTF-1 and napsin A for lung adenocarcinoma and oestrogen receptor, mammaglobin, GATA3 or GCDFP-15 for breast cancer [65]. The expression frequencies of different tissue- and organ-specific antigens in metastases and corresponding primary tumours are further outlined in **Table 4**.

Antigen	Tumour	Frequency, %	References
CDX2	Colorectal carcinoma	100	[66]
CDX2	Metastatic colorectal carcinoma	96.7–100	[67, 68]
SATB2	Primary colorectal carcinoma	96.0	[68]
SATB2	Metastatic colorectal carcinoma	92.2	[68]
CK20	Metastatic colorectal carcinoma	97.1	[68]
TTF-1	Lung adenocarcinoma	83.3	[69]
Napsin A	Lung adenocarcinoma	86.7	[69]
HMB-45	Metastatic melanoma	76–81	[70, 71]
MART-1	Melanoma	48.4–83	[72, 73]

Antigen	Tumour	Frequency, %	References
MART-1	Metastatic melanoma	63–82	[70, 72, 73]
Tyrosinase	Melanoma	71	[72]
Tyrosinase	Metastatic melanoma	63	[72]
PAX-8	Ovarian cancer	80	[69]
PAX-8	Endometrial cancer	100	[69]
PAX-8	Renal cancer	83–93.3	[69, 74]
PAX-8	Metastatic renal cancer	93.9	[74]
Napsin A	Renal cancer	50	[69]
Gross cystic disease fluid protein-15	Breast carcinoma	23.9–60	[75, 76]
Gross cystic disease fluid protein-15	Primary triple negative breast carcinoma	10–14	[75, 77]
Gross cystic disease fluid protein-15	Primary non-triple negative breast carcinoma	69	[77]
Gross cystic disease fluid protein-15	Metastatic triple negative breast carcinoma	21	[75]
Mammaglobin	Breast carcinoma	46.6–80	[75, 76]
Mammaglobin	Primary triple negative breast carcinoma	17–25	[75, 77]
Mammaglobin	Primary non-triple negative breast carcinoma	61	[77]
Mammaglobin	Metastatic triple negative breast carcinoma	41	[75]
GATA3	Invasive breast cancer	82.5–94	[76, 78]
GATA3	Primary triple negative breast carcinoma	20.2–87	[77–80]
GATA3	Metastatic triple negative breast carcinoma	44	[79]
GATA3	Luminal A breast carcinoma	99.5	[80]
GATA3	Luminal B breast carcinoma	97.7	[80]
GATA3	HER2-positive breast carcinoma	59.6–68.5	[76, 80]

CDX2, caudal type homeobox 2; SATB2, special adenosine-thymidine-rich-binding protein 2; CK, cytokeratin; TTF-1, thyroid transcription factor 1; HMB-45, melanosome protein human melanoma black 45; MART-1, melanoma antigen recognized by T cells 1; PAX-8, paired box gene 8; GATA3, guanosine-adenosine -thymidine -adenosine nucleotide sequences binding protein 3.

Table 4. Frequency of immunohistochemical expression of selected tissue- or organ-specific markers [66–80].

When differentiating between HCC and metastasis, the peculiar immunophenotype of fibrolamellar HCC must be recognised promptly. Fibrolamellar HCC expresses hepatocellular proteins, such as HepPar1, GPC3 or pCEA; biliary (CK7), progenitor and stem cell (CK19, CD44) antigens and macrophage markers (CD68). The granular or dot-like expression of CD68 in a tumour featuring appropriate morphology is helpful in diagnosing fibrolamellar HCC [6].

4. Molecular analysis

The molecular classification of hepatocellular carcinoma is still developing. Thus, different approaches have been proposed. Although the present tools of molecular analysis assure the

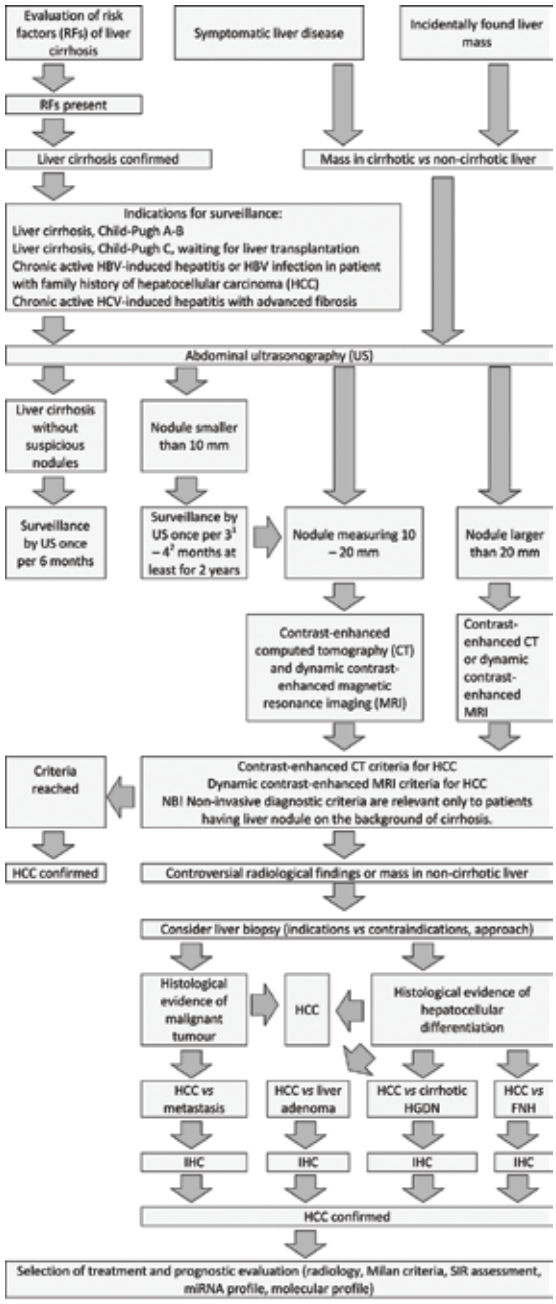

Figure 1. Diagnostic algorithm of hepatocellular carcinoma. 1—Recommended by the American Association for the Study of the Liver diseases (AASLD). 2—Recommended by the European Association for the Study of the Liver (EASL). Abbreviations: RFs, risk factors; *vs*, versus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; US, ultrasonography; CT, computed tomography; MRI, magnetic resonance imaging; HGDN, high-grade dysplastic nodule; FNH, focal nodular hyperplasia; IHC, immunohistochemistry; SIR, systemic inflammatory response; mi, micro; RNA; ribonucleic acid.

technical background for in-depth studies, HCC might be more difficult target for the systematisation of molecular findings than other tumours. The problems are associated with heterogeneity of etiological factors and their geographic distribution in different populations with diverse genetic background [81].

A trans-ancestry study has been carried out involving 608 cases of HCC. The cohort was created to reflect both etiological and geographic/genetic diversity of HCC. The main identified molecular targets were TP53–Rb pathway, Wnt pathway, modulators of chromatin and transcription, mTOR–PIK3CA pathway and mutations in genes regulating telomere maintenance [82].

French research team has recently proposed molecular classification into six subtypes, designated as G1–G6. The first three subtypes are characterised by *TP53* mutations and are high-grade tumours. G1–G2 share *AXIN1* and *ATM* mutations, while G1 also possesses *RPS6KA3* mutations. G3 is characterised by mutations in *TSC1*/*TSC2* and *FGF19*. G3 is also associated with haemochromatosis, macrovascular invasion, macrotrabecular and compact histological pattern as well as presence of multinucleated and pleomorphic cells. Sarcomatoid changes are more frequent in G1–G2, but clear cells—in G1. G4–G6 lack *TP53* mutations and are low-grade tumours. G5–G6 exhibit mutations in *CTNNB1* gene, while G4 lacks both mutations in *TP53* and *CTNNB1*. G4 tumours are more frequently characterised by small size, steatohepatitic morphology and inflammatory infiltrates as well as absence of satellite nodules and vascular invasion. G5–G6 carcinomas display microtrabecular pattern, cholestasis and lack inflammatory infiltrates. By immunohistochemistry, these HCCs are characterised by nuclear expression of beta catenin and strong positivity for glutamine synthetase [52].

5. Diagnostic algorithm of hepatocellular carcinoma

Nowadays, the classic diagnostic algorithm of HCC (see **Figure 1**) includes the evaluation of risk factors in a given patient to assess the need for surveillance. Cirrhotic patients are referred to ultrasound examination once per 6 months. Suspicious nodules are further evaluated by CT and MRI. Characteristic findings by CT and MRI including arterial hypervascularisation represent the basis of non-invasive diagnostics. In controversial and non-cirrhotic cases, biopsy is indicated that might need supplementation by immunohistochemistry according to the morphological features. Innovations are expected in the field of miRNA-based liquid biopsy to support radiological diagnosis, addition of SIR assessment and miRNA profile to select the optimal treatment, e.g. possibly broadening Milan criteria (see also chapter "Innovative Blood Tests for Hepatocellular Carcinoma: Liquid Biopsy and Evaluation of Systemic Inflammatory Reaction"), and novel immunohistochemical markers for cases that still remain ambiguous.

6. Conclusions

HCC is a frequent and aggressive malignant tumour, estimated to range sixth by incidence and second by mortality in the global cancer statistics. The high ratio of mortality to incidence (0.95)

and the close geographic correlation between incidence and mortality reflects the dismal prognosis. However, longer survival can be reached in early diagnosed and properly treated cases.

Awareness of the risk factors of HCC is helpful both in diagnostics and in order to set up the surveillance. Liver cirrhosis is the main risk factor; surveillance is indicated in these patients. A tumour found in cirrhotic liver is more likely to be HCC than metastasis or liver adenoma. However, the differential diagnosis includes a dysplastic cirrhotic nodule.

The other risk factors act mainly through inducing cirrhosis although a fraction of HCC can precede the development of cirrhosis in a patient affected by chronic liver disease or develop in non-fibrotic liver. Thus, the complete list of the risk factors of HCC includes chronic active hepatitis B or C, liver damage by alcohol and/or aflatoxins, as well as NASH. The risk factors can act synergistically. Evaluating the HCC risk in any patient, the relative risk must be considered in accordance to the risk factors that are identified in that individual. However, to estimate the expected cancer burden in the population, population attributable fractions are of importance; these parameters depend both on relative risk and population frequency of each particular factor.

Non-invasive radiological approach is the gold standard in the diagnostics of HCC in contrast with most of other malignant tumours necessitating confirmation by a biopsy. Biopsy is indicated only in radiologically controversial cases or to prove HCC in non-cirrhotic liver.

Ultrasonography is used for surveillance and the initial step of diagnostics. For surveillance of cirrhotic patient, US is carried out once in 6 months. If a suspicious focus is disclosed, the further approach is based on the size. Either CT or MRI is indicated for mass lesions larger than 20 mm, while both methods are recommended for a nodule measuring between 10 and 20 mm. Nodules that are smaller than 1 cm are followed up by US once in 4 months. Hypervascularity is a characteristic trait of HCC in CT and MRI. PET and CEUS may have additional role in HCC diagnostics.

If biopsy is carried out, HCC can be diagnosed if both signs of hepatocellular differentiation and cellular atypia or invasion are present. Low-grade tumours must be differentiated from dysplastic nodule, focal nodular hyperplasia and adenoma while high-grade HCC must be distinguished from metastasis. Mass lesion in cirrhotic liver is most probably a dysplastic nodule or HCC while adenomas and metastases usually develop in non-cirrhotic liver. In a Western patient, clearly malignant tumour in a non-cirrhotic liver has higher probability to represent a metastatic carcinoma.

Regarding immunohistochemistry, arginase-1 and HepPar antigen are reasonable hepatocellular markers that are used to distinguish HCC from metastases. Novel immunohistochemical markers of HCC include bile salt export pump protein and heavy chain of clathrin. Glypican should be used with caution due to the reported expression in a wide range of extrahepatic tumours. In order to discriminate between low-grade HCC and FNH, reticulin network, glypican-3 and heat shock protein 70 can be assessed. The differential diagnosis between high grade dysplastic nodule and low grade HCC can be very complicated as both processes share several morphological features and can coexist, biologically representing subsequent stages of HCC development. The features favouring malignancy over dysplastic nodule, include (1)

expression of at least two markers in a panel consisting from glypican-3, heat shock protein 70 and glutamine synthetase; (2) diffuse expression of CD34 due to higher oxygen tension in HCC and (3) loss of perifocal CK7- and CK19-positive ductular reaction as a sign of invasive growth.

Regarding molecular classification of HCC, reasonable success has been reached by French research group and trans-ancestry study team. However, no unified classification has been established yet. Molecular profile can have both diagnostic and prognostic value.

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Chapter 3

Innovative Blood Tests for Hepatocellular Carcinoma: Liquid Biopsy and Evaluation of Systemic Inflammatory Reaction

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Abstract

Hepatocellular carcinoma (HCC) is an aggressive tumour associated with dismal prognosis. To improve the outcome, early diagnostics is important. At present, classical HCC diagnostics is based on evaluation of risk factors, surveillance in cirrhotic patients, preference for non-invasive diagnosis by computed tomography or magnetic resonance imaging and biopsy confirmation in controversial cases. However, ambiguous radiological presentation, biopsy-related complications or insufficient representation of the pathology in the tissue core are well-known problems. Panel assessment of microRNAs has diagnostic and prognostic value; thus, in future, microRNA-based liquid biopsy could partially reduce the need for core biopsies. Systemic inflammatory reaction (SIR), characterised mainly by neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio and Glasgow prognostic score, may have prognostic value and can be incorporated in criteria for certain treatment approaches, e.g., becoming an adjunct to Milan criteria. Thus, innovations in HCC diagnostics are expected in the field of miRNA-based liquid biopsy for diagnosis/prognosis and SIR for prognosis/selection of treatment.

Keywords: hepatocellular carcinoma, HCC, liquid biopsy, miRNA, systemic inflammatory response, neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, Glasgow prognostic score, prognosis

1. Introduction

Hepatocellular carcinoma is one of the most aggressive human cancers. The total oncological mortality is decreasing in many developed countries, e.g., it has been reduced by 23% since 1991 in the United States of America (USA). In contrast, death rate of HCC is increasing, along with the incidence of this tumour [1, 2]. Positive changes are expected due to risk factor eradication by vaccination against hepatitis B and improved treatment of chronic hepatitis C. The treatment of HCC has also developed significantly, including radiofrequency ablation, transarterial chemoembolisation, liver resection and transplantation as well as molecular targeted treatment by sorafenib. However, SEER-based analysis has revealed that survival has improved in early but not in advanced cases [2]. Thus, timely diagnostics remain an important goal.

Most of the hepatocellular carcinoma cases develop on the background of liver cirrhosis or chronic inflammatory liver disease in precirrhotic stage, e.g. chronic viral hepatitis B or C, alcohol-induced or autoimmune liver disease or non-alcoholic steatohepatitis (NASH). This might facilitate the diagnostics by screening of the risk population. Nowadays, screening by ultrasonography and non-invasive radiological diagnosis by the means of computed tomography or magnetic resonance imaging is the mainstay of HCC diagnostics. However, the radiological findings in early cases can be difficult to interpret. Biopsy is indicated in such controversial cases.

However, the biological course of HCC can result in diagnostic difficulties even in biopsy. HCC frequently develops in a dysplastic cirrhotic nodule. Such early HCC is typically well differentiated. Over time, it progresses to advanced dedifferentiated HCC. The resulting heterogeneity can lead to diagnostic problems and failures in biopsy due to sampling errors. For instance, if a small nodule seemed suspicious but not overtly malignant by radiological imaging, leading to biopsy, the differential diagnosis between dysplastic nodule and HCC will frequently imply the necessity to distinguish between premalignant process and well-differentiated tumour, usually lacking marked cell atypia or clear-cut invasion. In addition, both processes can be adjacent in the tissues. Consequently, early diagnostics of HCC is not straightforward even in biopsy.

In addition, biopsy can cause complications, including arterial hypotension, bleeding, pneumothorax, haemothorax, haemobilia, acute pancreatitis, visceral perforation, biliary fistulas, sepsis and needle breakage. Arterial hypotension is seen frequently (1.1–4.0%), mostly due to vasovagal reaction. In few cases, falling arterial blood pressure might indicate bleeding, if it is unusually severe. Bleeding can develop in the liver tissues or in the peritoneal cavity. It is seen in 4.5% of patients and is more frequent if INR is elevated: frequency of bleeding was 3.3% in patients having INR 1.3–1.5 and 7.1% among those who had INR > 1.5. Pneumothorax and haemothorax have been reported in 0.35 and 0.18% of patients, if the needle has accidentally passed through lung or diaphragmatic and intercostal blood vessels. Haemobilia has been described in 0.1% of patients and can induce acute pancreatitis via biliary obstruction. The frequency of visceral perforation, biliary fistulas, sepsis and needle breakage is 0.01% each. In addition, biopsy can result in pain, experienced in up to 84% of patients. In 40% of cases, pain lasts for 24 hours. It is attributable to skin and liver capsule damage, viscerosomatic irradiation

(to shoulder) or complications that lead to peritoneal irritation (bleeding, bile accumulation in the peritoneum perforation of the internal organs). Few death cases have been reported after liver biopsy: 0.01–0.1% of patients [3].

Non-invasive means of HCC diagnostics would be beneficial. Two promising pathways include so-called liquid biopsy by microRNA analysis in blood and assessment of tumour-induced systemic inflammatory reaction (SIR). MicroRNA spectrum might have diagnostic and prognostic value. Regarding SIR, prognostic aspects have been studied and appear as promising adjunct how to select patients for treatment.

2. miRNAs in the diagnostics of hepatocellular carcinoma

MicroRNAs (miRNAs) are small, double-stranded, non-coding RNA molecules consisting of approximately 22 nucleotides. MiRNAs regulate gene expression at the post-transcriptional level [4, 5] acting as large-scale molecular switches. MiRNAs are found not only in cells but also in body fluids. Due to the stable and relatively simple structure, these molecules are good biomarkers for diagnostic and prognostic evaluation complying with the idea of so-called liquid biopsy—a patient-friendly blood test bringing the same information as a biopsy. In order to increase the diagnostic value of such tests, panels of miRNAs have been advocated. However, the biological course of HCC cause a possible pitfall in the elaboration of such diagnostic tests: as HCC mainly arise on the background of liver cirrhosis, inflammatory or metabolic liver diseases, these preceding pathologies can also alter the levels of miRNAs.

MiRNA-122 is attractive for its wide expression in liver tissues suggesting significant role in liver functions. MiRNA-122 is upregulated in serum of HCC patients and downregulated in HCC tissues suggesting specific excretion of miRNA-122 in blood by HCC. Although miRNA-122 shows high specificity and sensitivity for HCC in comparison with healthy controls (83.3 and 81.6%, respectively), levels of miRNA-122 change in other liver pathologies as well, limiting the diagnostic application [6].

Comparing HCC patients with those having hepatitis B or liver cirrhosis, serum levels of exosomal miRNA-18a, miRNA-221, miRNA-222 and miRNA-224 were increased, while miRNA-101, miRNA-106b, miRNA-122 and miRNA-195 were decreased. MiRNA-16 was decreased in HCC, and the levels significantly differed from those found in hepatitis C virus (HCV) infection because chronic viral hepatitis C and non-alcoholic fatty liver disease are characterised by the contrary changes—increase in miRNA-16. MiRNA-21 is characterised by positive characteristics in meta-analysis showing specificity and sensitivity for HCC diagnosis of 84.8 and 81.2%, respectively. The changes of miRNA-21 serum levels in HCC patients significantly differ from cases of chronic hepatitis; however, other malignant tumours can also yield higher serum concentration of miRNA-21 [7].

Several panels of miRNAs have been recommended. Exploring nine serum miRNAs (miRNA-21, miRNA-30c, miRNA-93, miRNA-122, miRNA-125b, miRNA-126, miRNA-130a, miRNA-193b and miRNA-222) in HCC and chronic viral hepatitis C patients, nine markers were decreased in chronic hepatitis C *versus* healthy controls, while seven markers (miRNA-21,

miRNA-30c, miRNA-93, miRNA-122, miRNA-125b, miRNA-130a and miRNA-222) were significantly ($p < 0.05$) decreased in HCC *versus* chronic viral hepatitis C patients and four (miRNA-93, miRNA-122, miRNA-125b and miRNA-130a) in HCC *versus* non-HCC patients [8]. Panel of three miRNAs (upregulated miRNA-92a-3p, downregulated miRNA-3126-5p and upregulated miRNA-107) could discriminate HCC from healthy controls [9] Evaluating serum levels of 13 miRNAs in HCV-associated chronic hepatitis, liver cirrhosis and HCC *versus* healthy controls, panel of three miRNAs (miRNA-122, miRNA-885-5p and miRNA-29b) in association with serum alpha-fetoprotein (AFP) level could identify HCC *versus* healthy persons, while four miRNAs (miRNA-122, miRNA-885-5p, miRNA-221 and miRNA-22) and AFP were recommended for HCC diagnostics in liver cirrhosis and two (miRNA-22, miRNA-199a-3p) along with AFP—in chronic hepatitis [10]. In another study of HCV-infected patients including cases of HCV-related chronic hepatitis, liver cirrhosis and HCC, serum levels of miRNA-126, miRNA-129, miRNA-155, miRNA-203 and miRNA-223 were significantly decreased in HCC *versus* non-HCC patients [11]. Panel of eight miRNAs was assessed in hepatitis B virus-infected patients diagnosed with HCC or liver cirrhosis as well as in healthy controls. The levels of hsa-miRNA-206, hsa-miRNA-141-3p, hsa-miRNA-433-3p and hsa-miRNA-1228-5p were significantly increased in HCC *versus* control group comprising both healthy and cirrhosis patients, while hsa-miRNA-199a-5p, hsa-miRNA-122-5p, hsa-miRNA-192-5p and hsa-miRNA-26a-5p were downregulated [4].

In addition to the diagnostic role, miRNAs have been evaluated in the prognostic aspect. The influence of miRNAs upon HCC stem cells has been exploited. It has also been suggested that miRNAs could become treatment targets [12].

3. Systemic inflammatory response

Many tumours, including hepatocellular carcinoma, evoke systemic inflammatory reaction (SIR). In the recent years, cancer-induced SIR has become an attractive research area as changes in blood cell counts or ratios or blood levels of certain proteins are associated with the biological potential, course and treatment response in many malignant tumours. Although complex pathogenesis lies behind these changes, SIR can be evaluated by simple, widely available and economically feasible blood tests.

SIR in cancer patients develops through local and central mechanisms. Locally, the invasive growth of malignant tumour injures surrounding parenchyma, connective tissues and endothelium. The tissue damage leads to inflammation necessitating supply of inflammatory cells from the bone marrow through the circulation. The production of acute phase proteins becomes upregulated as well. Endothelial injury activates platelets; indeed, the association between hypercoagulation and advanced cancers is classical. Tumour necrosis and hypoxia are additional causes of local inflammatory response. Cancer can also evoke immune response manifesting by local cellular reactions. In turn, the inflammation can have both tumour-enhancing and tumour-suppressing outcomes. The released cytokines and transcription factors can upregulate the proliferation of malignant cells. Release of metalloproteinases and other enzymes can promote tissue degradation facilitating invasion. Angiogenesis can be upregulated as well. The immune system, in turn, can limit growth of the tumour.

The systemic effects of cancer include alterations in bone marrow function, especially myelopoiesis. Besides the increased production and release of leukocytes, immature myeloid cells, including the precursors of granulocytes and monocytes, are retained in early stages of differentiation. Immature myeloid cells can act as immune suppressors and generate pre-metastatic niches, among other pathogenetic processes [13]. Thus, it has even been stated that cancer is an inflammatory disease [14]. Further, neutrophils can form neutrophil extracellular traps developed from externalised DNA network. These nets are bidirectionally associated with platelet activation and can contribute to cancer progression via several mechanisms; therefore, neutrophil extracellular traps also represent an attractive treatment target [15].

Cancer-related SIR involves cells of innate and adaptive immunity as well as soluble factors. Macrophages are recruited in tumour by hypoxia and tumour-released molecular agents including growth factors and cytokines [16]. Macrophage phenotype switch from tumour-suppressing classical M1 to tumour-promoting M2 subtype promotes angiogenesis and immunosuppression. Platelet activation contributes to cancer progression and patient mortality [15]. Neutrophils are locally recruited in the cancer via chemokine signalling. Neutrophil activation can contribute to angiogenesis and increased blood vessel permeability locally and metastatic spread systemically. In addition, immature myeloid cells and neutrophil extracellular traps might have tumour-promoting activity. These molecular events also highlight the association between infection or surgery-induced inflammation [17] and cancer relapse or metastatic spread. Thus, innate immunity is generally thought to act as tumour enhancers. In contrast, lymphocytes representing the adaptive immunity are considered to have tumour-suppressing effects [16], although contrary effects have been ascribed to certain subpopulations [18].

HCC can be considered a classical inflammation-induced cancer, as its most common risk factors are hepatitis B and hepatitis C virus infections. Inflammation is also present in liver tissues in patients affected by alcohol-induced hepatitis or NASH. Thus, SIR is not expected to have diagnostic value. Indeed, SIR parameters change before the tumour develops, e.g., increased NLR has been observed in chronic viral hepatitis C [19]. NLR is an independent prognostic factor in liver cirrhosis [20].

Wide variety of inflammation-based markers could be used as indicators of HCC prognosis, tumour recurrence and response to specific treatment. In particular, neutrophil, lymphocyte and platelet counts as well as C-reactive protein and albumin and their combinations, neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR) and Glasgow prognostic score (GPS) have prognostic value.

3.1. Neutrophil-to-lymphocyte ratio in HCC

Neutrophil-to-lymphocyte ratio is the most extensively evaluated parameter of SIR in HCC. NLR, calculated as the ratio between count of neutrophils and lymphocytes in blood, seems to reflect the intensity of systemic pro- and anti-tumour reaction. NLR has shown prognostic importance in surgically treated HCC cases, including early stage tumours; in liver transplantation; sorafenib treatment and different ablation techniques.

Several meta-analyses have been devoted to NLR in HCC patients. Thus, baseline NLR was associated with overall survival and recurrence-free survival, while post-treatment NLR was significantly associated with overall survival [21]. In meta-analysis of NLR in HCC patients

treated by liver transplantation, significant association with overall and recurrence-free survival was found [22]. In an early study, NLR was already confirmed as a prognostic factor in HCC. NLR was associated with overall and disease-free survival both generally and after different treatment approaches [23].

3.2. Neutrophil-to-lymphocyte ratio in surgically treated HCC

In HCC patients subjected to curative resection, several research groups have identified high NLR as an independent, significant predictive factor, associated with worse overall survival [24–27], shorter recurrence-free survival [24, 25, 28] and higher frequency of recurrence [29].

The studies show some variability in design, group size and cut-off level. However, the reported differences in survival are not only statistically significant but also biologically important. In 672 HCC patients treated by surgical resection, the recurrence rate within 1, 3 and 5 years was 77.4, 55.2 and 44.8% in those having low NLR (≤2.5), while the corresponding recurrence rates were 64.1, 45.2 and 35.5% ($p = 0.016$) in high NLR group [29]. In 303 patients who underwent surgical liver resection for HCC, high NLR (>2.0, based on ROC-detected cut-off) was significantly associated both with shorter recurrence-free and overall survival (both $p < 0.001$), but multivariate analysis confirmed it as an independent prognostic factor only for overall survival (hazard ratio (HR) 1.724; 95% confidence interval (CI): 1.241–2.394; $p = 0.001$). Huang et al. evaluated 1659 HCC patients, stratifying them by NLR quartiles. The 5-year overall survival rate was 60% in the lowest quartile contrasting with 27% in the highest quartile. NLR was associated by HR of mortality 1.031; 95% CI: 1.002–1.060; $p = 0.033$ [30]. In a large Japanese study, enrolling 958 patients who underwent hepatectomy, the 5-year survival rate was 72.9% in low NLR (<2.81) group *versus* 51.5% in patients with high NLR [24]. In another 256 Japanese patients, NLR was confirmed by multivariate analysis as an independent prognostic factor both for overall and recurrence-free interval. The respective hazard ratios were 2.59; 95% CI: 1.56–4.31; $p < 0.001$ and 2.11; 95% CI = 1.44–3.11; $p < 0.001$ [25]. In a smaller study of 113 patients, the recurrence-free survival was 42.4 months in patients having NLR < 3 but 7.9 months in those having NLR ≥ 3.0. The respective HR was 2.58; $p = 0.002$ [28].

The findings in Western patients have been less positive. In the largest Western series comprising 370 patients, treated in Memorial Sloan Kettering Cancer Center in New York, USA, PLR, but not NLR, was independently associated with worse recurrence-free survival and overall survival [31].

NLR retains prognostic value in several subgroups of surgically treated patients, including early cases. By multivariate analysis, preoperative NLR (at cut-off 2.8) was the strongest independent prognostic factor for overall survival after liver resection with curative intent for TNM stage I HCC. The HR was 2.69; 95% CI: 1.57–4.59; $p < 0.001$. The 5-year survival in high *versus* low NLR group was 45.0 *versus* 76.4%; $p < 0.001$. Interestingly, the association with survival in stage II or III was not significant, reflected by $p = 0.283$ and $p = 0.155$. In stage I patients, high NLR predicted more frequent extrahepatic recurrence ($p = 0.006$). As growth of HCC is associated with grade progression, these results suggest that NLR reflects the biological potential of HCC [32]. In Chinese cohort of 222 patients, preoperative NLR, using cut-off at

2.1, predicted overall survival in solitary small (≤5 cm) HCC after surgical resection and could discriminate outcome in patients having AFP levels not exceeding 400 ng/mL [33]. Further, in a large study of 963 HCC patients treated by potentially curative surgical resection, high NLR (>2.81) was an independent risk factor for overall and recurrence-free survival (both $p < 0.001$) in the general group as well as in early or intermediate stage HCC: Barcelona Clinic Liver Cancer (BCLC) stages 0/A or B (both $p < 0.05$) while no association was found in stage C [34]. NLR was also not associated with early (<1 year) mortality from cancer recurrence after liver resection for huge (at least 10 cm in diameter) HCC in 166 patients [35]. NLR was an independent factor that predicted ($p = 0.029$) early recurrence after curative resection of HCC presenting as a single focus in 193 Japanese patients [36]. However, contrary results have been reported as well, e.g., NLR had no prognostic significance in early HCC (BCLC stage 0/A) treated by surgical resection in 324 patients [37].

Somewhat contrasting data are reported regarding NLR in surgically treated patients with more advanced HCC. Although limited significance of NLR was previously noted in high-stage or large HCC, some authors have found significant role of NLR in advanced cases. Thus, in 81 hepatectomy-treated HCC patients with portal or hepatic vein tumour thrombosis, high NLR (defined as >2.9) was an independent prognostic factor for worse overall survival, characterised by HR 1.866; 95% CI: 1.048–3.322; $p = 0.034$. Significant association with recurrence-free interval was found as well. The overall survival in high *versus* low NLR groups was 6.2 months *versus* 15.7 months; $p = 0.007$, while the recurrence-free survival was 2.2 *versus* 3.7 months; $p = 0.039$ [38].

Not only baseline NLR but also the dynamic changes of NLR were found to be important. In 189 patients treated by curative resection for small HCC, the temporal change of NLR was an independent prognostic factor for overall (HR 2.637; 95% CI: 1.356–5.128; $p = 0.004$) and recurrence-free (HR 2.372; 95% CI: 1.563–3.601; $p < 0.001$) survival. The 1-, 3- and 5-year survival was 92.7, 70.0 and 53.0% in patients experiencing NLR increase *versus* 96.2, 87.5 and 75.9% in those with decreasing NLR ($p = 0.003$). High preoperative or postoperative NLR had lower prognostic value in this study [39]. Paralleling observations have been reported by Hung et al., studying patients with HCC recurrence after liver resection. The 5-year post recurrence survival was better (45.9%) in those continuously having NLR ≤ 2.5. Patients who had low NLR (≤2.5) at resection but high level (>2.5) at recurrence had 5-year survival of only 24.6%; the difference was significant as reflected by $p = 0.013$ [29].

3.3. Neutrophil-to-lymphocyte ratio in patients receiving transplantation for HCC

Different aspects of NLR have been evaluated in HCC patients subjected to liver transplantation, including transplantation from living donor. The prognostic value of NLR was revealed already in the early studies. Thus, NLR (applying the cut-off level at 5.0) was an independent predictor of overall and recurrence-free survival in 219 Italian patients after liver transplantation for HCC [40].

By univariate analysis, preoperative NLR was significantly associated by disease-free survival and overall survival in HCC patients after liver transplantation. The 1-, 3- and 5-year overall

survival rate in high *versus* low NLR group was 81.3, 56.7 and 51.0 *versus* 90.9, 74.2 and 66.8% ($p = 0.041$). Similarly, the 1-, 3- and 5-year recurrence-free survival rate in high *versus* low NLR group was 65.3, 48.5 and 39.4 *versus* 80.0, 68.0 and 65.2%. The difference was also significant as reflected by $p = 0.013$ [41]. Further, NLR was an independent prognostic factor for overall and recurrence-free survival after liver transplantation for HCC as was shown by multivariate analysis of 160 Western patients [42]. NLR was proved to be an independent risk factor for overall survival ($p < 0.001$) and recurrence-free survival ($p = 0.003$) in 248 male patients treated by liver transplantation [43]. Harimoto et al. reported on 213 patients receiving living donor liver transplantation for HCC. High preoperative NLR ≥ 2.66 was an independent predictor of recurrence [44].

NLR can be used in prognostic models to identify patients who exceed Milan criteria but still have good overall and tumour-free survival. In study reported by Wang et al., male patients were enrolled and thus the proposed models were verified in males only [43]. Combination of NLR and Hangzhou criteria has been suggested to identify patients who can be successfully treated by liver transplantation [45]. NLR has been included in the MORAL scores to predict recurrence after liver transplantation, and these scores were superior to Milan criteria [46]. Complex evaluation of NLR along with fibrinogen increases the prognostic accuracy in order to predict disease-free survival and overall survival in HCC patients treated by liver transplantation [41]. Pretransplantation NLR along with levels of C-reactive protein has been combined with Milan criteria to develop new selection criteria for living donor liver transplantation beyond Milan criteria [47].

However, contrasting findings have been published as well. Thus, NLR did not predict post-transplantation recurrence or worse overall survival in 150 patients within Milan criteria [48]. Limited prognostic impact of NLR was found in 124 patients who underwent living donor liver transplantation [49]. In Western patients, NLR was not predictive of treatment success regarding liver transplantation or other tested approaches (hepatectomy, transarterial chemoembolisation). Although the group was quite small (75), Child-Pugh and Model for End Stage Liver Disease (MELD) scores were informative [50].

3.4. Neutrophil-to-lymphocyte ratio in sorafenib-treated HCC patients

In patients with unresectable HCC, treated by multikinase inhibitor sorafenib, high NLR (>3.1) was a significant independent prognostic factor, associated with worse overall survival. Better treatment response was observed in patients with low NLR [51]. The findings were confirmed by another study, reporting on 442 sorafenib-treated patients (Japan, Italy and United Kingdom) with advanced HCC. High NLR again was an independent prognostic factor, predicting shorter survival with HR 1.218; 95% CI: 1.108–1.322; $p < 0.0001$ [52].

Regarding combined approach, high pre-treatment NLR (>3.0) was an independent predictor of worse overall survival in 40 patients with unresectable HCC treated by transcatheter arterial embolisation and sorafenib. The median survival in high *versus* low NLR group was 14 months (95% CI = 10.1–17.9) *versus* 26 months (95% CI = 17.4–34.6). The difference was significant ($p = 0.001$) and biologically remarkable [53]. Other researchers have also confirmed that NLR was independent predictor of overall survival in patients with advanced HCC treated by sorafenib [54].

3.5. Neutrophil-to-lymphocyte ratio in HCC patients undergoing tumour ablation

NLR has been investigated in regard to different embolisation and ablation techniques. High NLR (>3) predicted significantly worse treatment results ($p = 0.014$) and early disease progression ($p < 0.0001$) in 86 treatment-naive patients subjected to arterial chemoembolisation or radioembolisation [55]. Elevated pre-treatment NLR (>1.85) was associated with overall survival and disease-free survival in 178 HCC patients subjected to transcatheter arterial chemoembolisation (TACE). The median survival in high *versus* low NLR group was 8 *versus* 17.5 months. The 1-, 3- and 5-year overall survival rates in these groups were 42.1, 19.6 and 9.5 *versus* 57.3, 44.1 and 27.2%, respectively ($p < 0.001$). Differences in disease-free survival were significant as well ($p < 0.001$). Multivariate analysis confirmed NLR as a significant ($p = 0.04$), independent prognostic factor for survival after TACE [56].

In patients with advanced HCC treated by hepatic arterial infusion chemotherapy, high NLR was a significant predictor of lower response rate, worse progression-free and overall survival [57]. Baseline NLR was a significant predictor of treatment response and progression-free survival after hepatic arterial infusion chemotherapy for advanced HCC [58]. In patients receiving hepatic arterial infusion chemotherapy by cisplatin and fluorouracil, response rate and overall survival were associated with NLR [59].

Dynamic changes of NLR had independent prognostic significance ($p = 0.035$) in HCC with portal vein tumour thrombosis treated by microwave ablation after transarterial chemoembolisation [60]. In 506 patients treated by thermal ablation of recurrent HCC, high pre-treatment NLR (≥2.14) was a prognostic factor for recurrence-free survival, confirmed by Cox multiple regression analysis. The 1- and 3-year recurrence rates in high *versus* low NLR groups were 57.9 and 82.5 *versus* 20.7 and 31.6%. The difference was statistically significant, confirmed by $p < 0.001$ [61].

Pre-treatment NLR was associated with worse overall survival in early HCC after radiofrequency ablation. Post-treatment NLR was associated both with worse overall survival and recurrence in early HCC after radiofrequency ablation [62]. Similarly, NLR dynamics, but not pre-treatment NLR, was an independent prognostic factor for overall survival and recurrence-free survival in patients with small HCC treated by radiofrequency ablation [63]. In patients treated by radiofrequency ablation for HCC, post-treatment NLR was associated with recurrence and survival. Pre-treatment NLR was associated with recurrence only in patients who had HBV infection and HCC but not in those who developed HCC in association with HCV infection [64]. In unresectable HCC treated by radioembolisation, elevated NLR was an independent predictor of worse survival [65].

3.6. Neutrophil-to-lymphocyte ratio and tumour characteristics

NLR has been mostly assessed in correlation with survival or treatment response. However, some observations are reported on the association between systemic inflammatory response and tumour morphology in gross and microscopic level. Thus, high NLR is observed in patients having larger tumours, multiple HCC foci, higher grade of HCC and vascular invasion [29]. In few studies, the infiltration of neutrophils and macrophages in liver tumours has been assessed. Peritumoural tissues are characterised by higher ratio between neutrophils

and T lymphocytes, and higher ratio also correlates with lower overall survival. The combination of these findings suggests that neutrophils might facilitate tumour progression. They suppress adaptive immunity by death ligand expression [66]. Correlation between NLR and PD-L1 expression in the centre of tumour but not peritumoural tissues has been described [67]. High NLR was associated with CD163-positive tumour-associated macrophages [24]. High NLR was associated with higher peritumoural but not intratumoural CD163 and IL-17-expressing cells [68].

3.7. Platelet-to-lymphocyte ratio in HCC patients

Platelet-to-lymphocyte ratio (PLR) is another frequently assessed estimate of SIR, although fewer publications have been devoted to PLR than to NLR. Nevertheless, prognostic role of PLR has been evaluated in different aspects of HCC patient treatment, including surgery [31, 69, 70], transplantation, sorafenib treatment, TACE and ablation techniques.

In the largest Western series enrolling 370 HCC patients, treated by surgical resection, higher preoperative PLR was identified as an independent risk factor of worse overall survival and recurrence-free survival [31]. Other research groups have confirmed the association between PLR and prognosis. Higher preoperative PLR is an independent predictor of worse overall survival in HCC patients undergoing curative liver resection [69, 70] as was shown in two large Eastern cohorts comprising 778 [70] and 1804 [69] patients, respectively. The significant association with overall survival ($p < 0.001$) was retained in specific subgroups, e.g., patients having cirrhosis or being positive for HbsAg [70]. In some studies, the associations between PLR and prognosis were statistically significant but not independent. Thus, PLR was significantly associated with disease-free and overall survival in 332 HCC patients after hepatectomy [71]. The association between higher PLR and shorter recurrence-free survival in HCC patients undergoing curative liver resection was statistically significant but not independent [70]. Finally, no association has been found in some studies. Hence, in 113 HCC patients undergoing curative resection, PLR was not confirmed as a significant prognostic factor for recurrence-free survival. NLR was found to be superior in this study [28].

PLR has been analysed in the context of liver transplantation. Already in early studies, statistically significant association was found between pretransplantation PLR and cancer recurrence after liver transplantation. In 146 patients, the recurrence-free survival was 80.7 *versus* 91.6%; the difference was statistically significant as confirmed by $p = 0.02$ [72].

In 122 Chinese patients undergoing transarterial chemoembolisation (TACE) for HBV-related HCC, high pre-treatment PLR (≥96.13) was an independent, statistically significant ($p = 0.001$) factor that predicted worse survival [73].

In 414 patients affected by recurrent HCC and treated by thermal ablation, high pre-treatment PLR (≥87.87) was associated with higher risk of recurrence and worse recurrence-free survival. The 1- and 3-year recurrence rates in high *versus* low PLR groups were 56.0 and 79.5 *versus* 39.9 and 54.8%; $p < 0.05$ [74].

Regarding sorafenib-treated cases, PLR has not shown prognostic value in patients receiving sorafenib for advanced HCC. Although the negative result could be attributed to the small group size (16 patients), significant association with NLR was still confirmed [75].

Several meta-analyses have recently been carried out to evaluate PLR in hepatocellular carcinoma. Significant association between higher PLR and increased risk of death was reported by Hu and Yu, reporting odds ratio for death 1.59; 95% CI: 1.15–2.20 on the basis of meta-analysis of six studies with 1446 HCC patients [76]. The association between high PLR and worse overall survival was confirmed by Ma et al., Song et al., and Zhao et al. [77–79]. In a meta-analysis of nine studies including 2017 patients, high PLR was associated with poor overall survival (HR 1.63; 95% CI: 1.42–1.88; $p < 0.001$) as reported by Ma et al. [77]. Evaluating 2507 patients in 11 studies, high PLR was significantly associated with worse overall survival, as reflected by hazard ratio HR = 1.78; 95% CI: 1.36–2.34; $p < 0.001$ [78]. In a meta-analysis of 10 studies including 2315 patients, high PLR was associated with the HR for worse overall survival of 1.60; 95% CI: 1.23–2.08; $p = 0.0005$ [79].

Controversial findings are reported regarding PLR and recurrence-free survival. The association has been confirmed by some research groups [77, 78] but denied by others. In a meta-analysis of nine studies including 2017 patients, high PLR was significantly ($p < 0.001$) associated with poor recurrence-free survival: HR = 1.32; 95% CI: 1.15–1.52. In a meta-analysis of 11 studies comprising 2507 patients, elevated PLR was significantly associated with worse recurrence-free survival, as reflected by hazard ratio HR = 1.82; 95% CI: 1.56–2.13; $p < 0.001$. In contrast, by meta-analysis of 2315 patients in 10 studies, high PLR was not significantly associated with worse recurrence-free survival as the HR was 1.21; 95% CI: 0.87–1.67; $p = 0.26$ [79].

High PLR also showed correlation with high tumour size exceeding 3 cm, TNM stage, lymph node metastases and distant metastases [78]. The findings regarding vascular invasion are controversial again, as the association is confirmed in some studies [78] but not others [77]. No association between PLR and tumour multifocality and higher grade has been confirmed [77].

3.8. Glasgow prognostic score in hepatocellular carcinoma

The prognosis and treatment options of HCC patients depend not only on tumour progression but also on the extent of liver dysfunction. As a consequence, several staging systems have been proposed to predict prognosis for HCC, including Glasgow prognostic score (GPS), based on the levels of C-reactive protein and albumin (**Table 1**). GPS in HCC patients with hepatocellular carcinoma is an independent prognostic predictor after hepatic resection, with higher score indicating worse prognosis [80]. Thus, among 144 patients who underwent surgical resection for HCC, GPS 2 was associated with worse disease-free (HR = 2.527; 95% CI: 1.163–5.490; $p = 0.019$) and overall (HR = 8.012; 95% CI: 2.818–22.784; $p < 0.001$) survival, but GPS 1 with shorter overall (HR = 2.277; 95% CI: 1.029–5.039; $p = 0.042$) survival than seen in patients preoperatively presenting with GPS 0 [80]. The independent prognostic role of GPS for overall survival was confirmed by other research teams [81] and meta-analysis [82]. Modified GPS score and dynamics of GPS score have also shown prognostic value. Thus, elevated modified GPS was associated with overall survival (HR = 2.21; 95% CI: 1.73–2.82; $p < 0.05$) in a meta-analysis of 2047 HCC patients [83]. Dynamics of GPS (assessed in association with hepatitis B infection status) was an independent predictor of overall survival in 247 patients treated by liver resection [84]. In addition, GPS was related to blood transfusion requirement and postoperative pulmonary complications after liver resection for HCC [85]. In patients undergoing liver transplantation for HCC, elevated GPS, reaching 1 or 2, was

Score	Definition
Glasgow prognostic score	
0	C-reactive protein (CRP) < 10 mg/L AND albumin ≥35 g/L
1	One high-risk finding: CRP ≥ 10 mg/L OR albumin <35 g/L
2	Both high-risk findings: CRP ≥ 10 mg/L AND albumin <35 g/L
Modified Glasgow prognostic score	
0	CRP ≤ 10 mg/L irrespective of albumin level
1	Increased CRP on the background of normal albumin level: CRP > 10 mg/L AND albumin ≥35 g/L
2	Increased CRP and hypoalbuminaemia: CRP > 10 mg/L AND albumin <35 g/L

Table 1. Glasgow prognostic score and its modifications [30].

significantly associated with poor overall (p = 0.018) and recurrence-free (p = 0.030) survival. Complex scoring system has been created on the basis of Milan criteria and GPS [86].

Regarding advanced HCC, GPS retained independent prognostic value for survival (HR = 1.410; 95% CI = 1.060–1.874; p = 0.018). In addition to the statistical significance of results, the biological survival differences by GPS were also remarkable, e.g., median survival was 480 days in patients presenting with GPS 0 and 154 days in those having GPS 1 or 2 [87]. GPS was an independent prognostic factor (HR = 5.483; 95% CI: 2.563–11.729; p < 0.001) for overall survival in patients undergoing sorafenib treatment: the median survival in those having GPS 0 *versus* elevated GPS 1 or 2 was 18.1 *versus* 5.2 months; p < 0.001 [88].

In HCC patients undergoing transarterial chemoembolisation, GPS was an independent prognostic factor for overall survival (HR = 1.697; 95% CI: 1.325–2.174; p < 0.001). It was also found to be superior to other inflammation scores as NLR, PLR or modified GPS. Based on these findings, the authors proposed a new complex score that included Child-Pugh stage, number of tumour nodules and proportion of affected liver, AFP, presence or absence of portal vein thrombosis and GPS. The new score was also a significant predictor of survival as the HR was 1.724; 95% CI: 1.347–2.285; p < 0.001 [89].

4. Conclusions

HCC is a frequent and aggressive malignant tumour. Longer survival can be reached in early diagnosed and properly treated cases. In the future, diagnosis of HCC might be based on combination of radiological features and miRNA profile representing liquid biopsy. MiRNA profile can have both diagnostic and prognostic value. Thus, further lowering of the needs for core biopsy might be expected, leading to patient-friendly examination and avoiding the rare but still possible complications. After the diagnosis of HCC has been confirmed, SIR assessment by NLR and PLR represents innovative means of prognostic evaluation.

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Chapter 4

Cellular Senescence and Their Role in Liver Metabolism in Health and Disease: Overview and Future Directions

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Additional information is available at the end of the chapter

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Abstract

Chronic liver disease has globally risen mainly due to a prevalent hepatitis C virus (HCV) infection rate and an epidemic of obesity. It is estimated by the year 2030, 2.2 billion people around the world will be overweight and 1.1 billion people will be obese. Diabetes and obesity are the main risk factors for the development of the metabolic syndrome and in the liver of non-alcoholic fatty liver disease (NAFLD) which could progress to non-alcoholic fatty steatohepatitis (NASH) related cirrhosis and liver malignancy. At present there is not effective therapy for NASH besides loss of weight and exercise. Furthermore, optimal management of HCC with curative intent includes resection or liver transplantation. Nevertheless, these therapies are limited because the degree of liver dysfunction or the medical conditions at the time of diagnosis and the scarcity of available liver grafts. The role of cellular lipid management and metabolism in human health and disease is taking a center stage. The present overview articulates the current pathophysiology of fatty liver disease under the aging processes, potential biological markers of liver disease diagnosis and progression and future therapies.

Keywords: review, senescence, cell aging, mitochondrial function, cancer, obesity, NASH, ROS, lipids, metabolism

1. Introduction

1.1. Global burden of chronic liver disease

Chronic liver disease has globally risen due to mainly a prevalent hepatitis C virus (HCV) infection rate and an epidemic of obesity [1–7]. During the last 2 decades, global viral hepatitis

has increased by 163% (from 0.89 million to 1.45 million) and in 2013, viral hepatitis infections became the 7th leading cause of death [7]. While HBV infection is decreasing in most endemic areas due to successful vaccination policies, HCV infection lacks the benefits of a vaccine [7]. Even though HVC antiviral therapies recently introduced in clinical practice are highly successful, its implementation is limited due to access and/or financial constraints. Morphological studies in HCV showed hepatocyte lipid accumulation similar to the one that occurs in obesity.

Our group has estimated that 2.2 billion people will be overweight and 1.1 billion people will be obese globally by 2030 [8, 9]. In addition, 36.1% of adult men and 32.4% of adult women were diagnosed with the metabolic syndrome in 2010 [10]. Obesity represents the core component of the metabolic syndrome, a cluster of metabolic disarrangements including dyslipidemias, insulin resistance status, hypertension, diabetes and organ metabolic disturbances such as non-alcoholic fatty liver disease (NAFLD) and its inflammatory component non-alcoholic fatty steatohepatitis (NASH), nephropathy, cardiomyopathy and muscle dysfunction [10]. Hepatocellular carcinoma (HCC) has been reported more often in non-cirrhotic livers in the background of NASH and its risks factors include male gender, older age, cigarette smoking, obesity and insulin-resistant states [3, 4, 11]. Overweight and obesity are associated with an overall increase in liver primary cancers of 17% and 89%, respectively [2, 11, 12] and males with a BMI > 35 had a 3.5–4 increase risk of liver malignancy [12] (**Figure 1**). Optimal management of HCC with curative intent includes resection or liver transplantation. Nevertheless, these therapies are limited because the

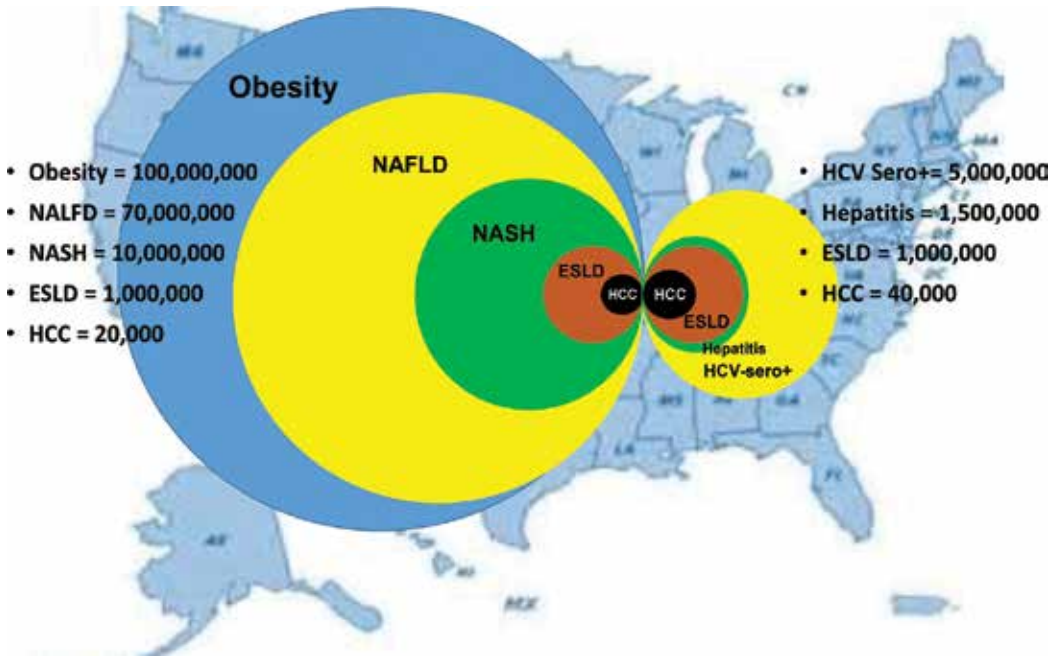

Figure 1. *Main causes of end stage liver disease (ESLD) and hepatocellular carcinoma (HCC).* Hepatitis C virus infection (HCV) and obesity are the main cause of the global and Western increase in ESLD and HCC. In USA 1 out of 3 adults is overweight and approximately 5 M people is HCV seropositive. While prevention relays in stopping virus transmission and implementing programs of healthy caloric intake and exercise programs, treatments of established ESLD and its malignant consequence are similar. Nevertheless, the most effective surgical treatment, liver transplantation is limited due to scarcity of donors and loco-regional therapies have limited survival effect due to malignant recurrence or progression of liver dysfunction.

degree of liver dysfunction or additional medical conditions at the time of diagnosis and the scarcity of available liver grafts. The role of cellular lipid management and metabolism in human health and disease is taking a center stage [13]. Higher fat intake, lower physical activity and a progressively aging population are among the behavioral and social factors of this phenomenon that add to the genetic load [1]. An overview of the role of cell aging and senescence in liver metabolic responses to high caloric intake will be performed in the pages to follow.

1.2. Regeneration, necrosis, apoptosis and senescent: a constant changing balance

The liver is a unique organ with an innate ability to regenerate through mass compensation to satisfy portal flow and metabolic demands [14]. After injury and cell necrosis, immune recall of resting cells occurs and activation of oval-precursor cells in conjunction with platelets migration switch to a cell division renewal cycle [15, 16]. Mitosis is more prominent at the peri-portal stem cell niche site (zone 1) assuring clonal expansion until reaching zone 3 (peri-central vein) [15, 16]. In health, liver mass homeostasis is closely regulated through a delicate balance among regeneration, apoptosis (programmed cell termination), and senescence. During states of acute liver injury, the pendulum moves towards a regenerative and repair phase, however, during chronic states of liver injury collagen synthesis and deposition persists leading to organ fibrosis. In addition, natural processes of organ aging play a main role in organ response to both acute and chronic injuries. Primary cell life span is determined by a limited number of cell duplications, the so called Hayflick limit [17]. After such limited divisions, cells enter a state of cell replicative senescence which is believed to be triggered by shortening of telomere ends. Replicative cellular senescence is a stable form of cell arrest characterized by a lack of cell proliferation activity and apoptosis resistance mediated through a lack of mitogen response even though the cells remain metabolically active. On the other hand, cells can be induced to a senescence status by a variety of cellular stressors such as DNA damage, UV light, radiation, oncogene activation, increased H_2O_2 production and heat stress [17, 18]. Senescent cells undergo morphological changes as they acquire an enlarged and flattened morphology, in addition to an increase expression of the senescence associated markers ß-galactosidase (SA-ß-GAL), an accumulation of the senescence associated heterochromatic foci (SAHF) and DNA damage foci, and the expression of the senescence associated secretary phenotype (SASP) [18]. Senescent status is achieved and maintained by active signaling of p53, a tumor suppressor gene that exercises its effects through activation of p21, a potent cell cycle inhibitor, and the p16-retinoblastoma protein [18]. Cells induced into an irreversible cell cycle arrest at the G1 phase will undergo metabolic disturbances with an increase reactive oxygen species (ROS) production, decrease adenosine triphosphate (ATP) synthesis and accumulation of lipofuscin [17].

Changes in the content of daily oral intake can influence life span and thus cell aging. The cell death-inducing DNA fragmentation factor α-subunit-like effector A (Cidea), is a transcriptional coactivator implicated in lipid accumulation, cell stress and cell aging. Authors showed in rodents, that a high lipid diet up-regulated Cidea with hepatic lipid accumulation, cell stress, mitochondrial dysfunction and genetic upregulation of aging [19]. Other studies, in support of this findings have shown a life span reduction up to 30% in genetically obese mice (ob/ob) and this reduction was reversed by a caloric restricted diet [19]. Lipid enriched diets are associated in humans with DM type 2, HTN and cardiovascular events all

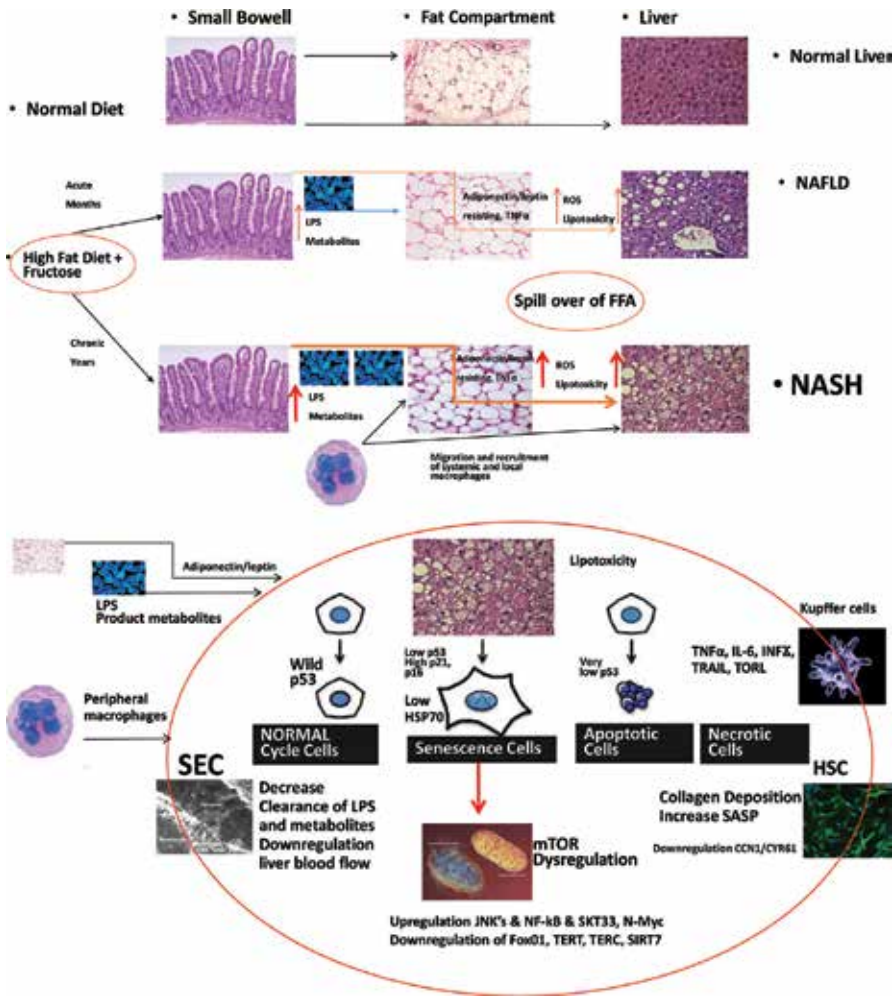

Figure 2. (A) *Morphological changes observed in the mice model of high fat diet (HFD) plus fructose (Western Diet) in the microbiota, fat content tissue and liver.* Liver cells accumulate FA in form of TG from the spill-over of lipid excess in the fat compartment and after saturated the normal processes of liver lipid metabolism. (B) *Local liver inflammatory response from lipid excess.* Lipotoxicity an addition to increase LPS activates SEC, HSC and Kupffer cells inducing more parenchymal cells into senescence and apoptosis which changes the local mielue into an inflammatory microenvironment. Continuous HFD decreased further mitochondrial function with lower ATP production and increase collagen deposition leading to progressive liver fibrosis, liver dysfunction. The state of progressive liver fibrosis due to a local and systemic inflammatory state results in an increasing insulin resistance status with the full metabolic syndrome phenotype. Its progression results in decompensated ESLD and the development of malignancy.

of which limit life span. Caloric restriction without malnutrition can extend life span while caloric excess has the opposite effect [20]. Thus, the choice of oral intake has a profound impact on life span.

The free radical intermediates hypothesis of cell aging still remains the most reasonable in the induction and maintenance of the senescent status [17, 21]. ROS, reactive nitrogen sp., and lipid peroxide are important regulators of cell signaling that provides reliable maintenance of

cellular components, support redox-state and regulate the function of highly metabolic active cells as in hepatocyte and immune cells [20, 21]. ROS in excess from over-oxidation of lipids, proteins, nucleic acids and other macromolecules is associated with a misalignment of their functional activity, reactions that if they last through the cell cycle can lead to permanent cell dysfunction and/or accelerated aging cell process. Thus, an excess of food intake in form of continuous lipid charge will test the oxi-redox systems that keeps the fragile mitochondrial equilibrium in balance. Continuous metabolic stress changes the equilibrium towards lower levels of antioxidants (glutathione sp.) with further increase of ROS that in turn accelerates processes of apoptosis and senescence. Former processes further lead to arrest of regeneration and activation of hepatic stellate cells (HSC) and therefore fibrogenesis (**Figure 2A**).

The hormonal mielau modulates cellular response to caloric intake. Insulin and somatotropin signaling are critical not only in the control of cell aging and longevity under conditions of abundant food supply but also in mediating the effects of caloric restriction on life span. In a rodent model of thyroxine induced aging, thermogenesis was directly correlated with increased mitochondrial function, increased ROS production, decreased concentration of glutathione reduced, downregulation in the activity of antioxidants enzymes and increased senescent marker expression in the liver as well as in other organs [21]. Estrogen influences lipid metabolism through nuclear receptors which enhances apoptosis of mutated cells, improves mitochondrial function, and decreases the metabolic syndrome phenotype [10, 22]. Actions that may explain, at least in part the constant disparity of overall life expectancy by gender.

Lifestyle changes such as exercise and caffeine supplementation have shown to increase the ratio of reduced/oxidized glutathione in liver and muscle tissue in the rodent model [23]. Although liver enzymes were identical in experimental and controls groups, plasma levels of cytokines associated with inflammation (IL-1ß, IL-6, TNF-α and INF-α) and cell aging were found to be significantly decreased in the experimental group when compared to controls [23]. It was noted that although exercise increased the production of ROS, exercise also evoked a beneficial increase in levels of cell antioxidants, and lowered levels of oxidative damage when cells were exposed to a second injury, i.e. lipid charge. Thus, the concept of exercise inducing gene expression of antioxidant enzymes that may protect the cell from other insults was called 'hormesis' [23]. Although caffeine, a member of the methyl-xanthine family increased the ratio of reduced-oxidized glutathione, no other markers of cell stress were modified. Perhaps, caffeine potentiates further the beneficial effects of exercise.

2. Liver metabolism in health and disease

The reduced tri-peptide glutathione (GSH) is the major antioxidant in the body responsible for maintaining the intracellular redox balance. 90% of the GSH in plasma derives from the liver [24] and aging is associated with a progressive decline in the levels of GSH in humans and rodents [25]. Senescent liver cells in culture showed elevated ROS leading to a state of chronic oxidative stress. In addition, age associated decline in GSH has been linked to an activation of neural sphingolipid hydrolase enzyme (NSMase) and the accumulation of bioactive ceramide, a precursor of inflammation [25]. The availability of L-cysteine is the rate-limiting

factor of GSH synthesis and oral supplementation of cysteine alleviates GSH deficiencies in humans and rodents [25]. GSH deficiency can be alleviated by the oral intake of cysteine and its restoration rates appears to be age and sex dependent. Older animal models are associated with increased cellular stress and an enhanced subcellular injury after heat stress associated with an increased iron intracellular deposition [26]. These cause damages to mitochondria and lysosomes. Although a more precise mechanism of organelle damage was not enunciated, iron deposition mediated a decrease in Transferrin-receptor-1 which upregulates the iron storage protein ferritin after heat stress. Nevertheless, the synthesis of the iron exporter protein ferroportin was delayed [26]. Effect that may explain at least in part, organelle damage in the aging cell that occurs after natural oxidants depletion (**Figure 2B**).

A diet enriched in calories and lipids increases free fatty acids (FA) in plasma obligating cells to protect themselves from lipotoxicity or death by either oxidizing FA's or sequestering them as triacylglycerol (TAG) within lipid droplets (LD) [13]. PGC-1, an exercise-induced transcriptional coactivator may play an important role in coordinating intra-muscular LD-signaling with mitochondrial remodeling. TAG within lipid droplets are the major form of energy storage in the body (muscle, liver, fat tissue) and a reservoir of membrane lipid component. TAG synthesis is initiated by glycerol-3-phosphatase acetyltransferases (GPAT) at the mitochondrial and sarcoplasmic reticulum membranes and it is completed at the sarcoplasmic reticulum by the sn-1-acyl-glycerol-3-phosphatase acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP) and sn-1,2-diacylglycerol acyltransferase (DGAT) [13]. Synthetized LD-TAG are localized preferentially in proximity to mitochondrial membranes named "contact zones". Once TAG's are released, they are mainly used in the mitochondria for ATP synthesis via oxidative phosphorylation from the ß-oxidation path. The "athlete paradox" states that the accumulation of TAG in the trained and insulin sensitive cells is in greater proportion than the TAG accumulation in cells from diabetic subjects with insulin resistance. This observation supports the hypothesis of mitochondrial dysfunction as a factor of TAG accumulation from a sustained lipid charge.

The protein family of perilipins (Plin) is associated with LD's and their scaffolding may affect the interaction between TAG and the mitochondria [13]. The Plin family consists of Plin1 to 5; the most common PAT (perilipin/ADRP/TIP47) interacts with LD in different proportions. In the liver, down-regulation of Plin2 promotes a reduction of hepatic steatosis and increases insulin sensitivity, albeit a reduction in both Plin2 and Plin3 is associated with insulin resistance [13]. In the heart, a Plin5 deficiency causes increased lipid oxidation, increased ROS production and decreased cardiac function. In heart and skeletal muscle TAG and FA are the main metabolic source of energy through the ß-oxidation pathway, suggesting a very tightly regulated process from cell storage to mitochondrial metabolic use. While TAG may come from LD, FA's are mainly transported in plasma as albumin-bound or as part of the very low density lipid-protein (VLDL) complex. Different transmembrane transporter systems are involved in their translocation to the inner cell compartment where the long chain fatty acid (LCFA) forms thioesters with coenzyme A (CoA). LCFA-CoA can form TAG for storage as LD, or can enter the outer mitochondrial membrane where CPT1 catalyzes the reaction of LCFA-CoA to LC-acylcarnitine. The former compound can actively cross the inner mitochondrial membrane with the exchange of carnitine for acylcarnitine. CACT is highly expressed in tissues with predominant ß-oxidation metabolism.

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2.1. In Health

The metabolism of FA in the mitochondrial matrix is sequentially catalyzed through a ß-oxidation process by four enzyme families: acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. While acyl-carnitine is converted back to acyl-CoA to enter the TCA cycle, dehydrogenases activity shows different affinity for short, medium, long and very long FA's [13]. Every cycle of ß-oxidation renders acyl-CoA and shortens the FA chain by 2 carbons providing the equivalents of electron donors NADH and $FADH_2$ which is the driving force for the synthesis of ATP. Although the ß-oxidation pathway is an effective way of ATP production, an overload of FA may play the role of un-coupler that exercises an inhibitory effect on the respiratory chain through a proton-phoric effect on the inner mitochondrial membrane. This effect results from the implicit effect of FA on mitochondrial membrane porosity by opening of the permeability transition pores, which results in the subsequent loss of electrical gradient and arrest of the respiratory chain [13]. The above concept favors the hypotheses of mitochondrial dysfunction from FA overload as the primary step of the insulin resistance state in obese patients. Other authors have found no mitochondrial respiratory changes in the steatotic liver but in mitochondria from skeletal muscle from a rodent model of high fat diet induced obesity [27]. Authors have proposed mitochondrial changes are due to an adaptation of the mitochondria to the high lipid charge rather than a defect per se in its function. No explanation was provided by the authors regarding the increase in ROS and inflammatory changes observed. Others suggested a protected effect from caloric lipid surplus against the development of metabolic dysfunction, as long as cells maintained functional adipocyte storage with low levels of tissue inflammation [28]. Once adipose accumulation saturates cell capacity, fatty excess spills over into other tissues leading to LD accumulation with subsequent lipid oxidation and an inflammatory response which precedes the metabolic syndrome manifestations [28]. The Delete in Breast Cancer-1 (DBC-1) protein is an important regulator of fat accumulation and storage in fat tissue which exercises its action by inhibition of SIRT1. In the DBC-/KO mice exposed to HFD, it was observed high plasma levels of FA with no liver steatosis, lower expression of senescence cells and increased storage of FA in the adipocytes with no development of insulin resistance [28]. Thus, DBC-1 protected liver and adipocytes from senescence by preservation of the fat compartment function with liver sparing and insulin sensitivity. Interestingly, a comparison has been made between this rodent model and the so called "healthy obese subjects" where there is fat accumulation but no signs of metabolic syndrome or systemic inflammation.

2.1.1. Metabolism and inflammation

Obesity depresses the anti-inflammatory effects of the heat shock proteins (HSP70) pathway, an inhibition that may contribute to the progression from NAFLD to NASH [29]. Excess of lipids and fuels trigger a low grade inflammatory response in both fat and liver tissues that correlates with the impaired insulin responsiveness. TBARS, a simple but fair estimate of lipoperoxidation/malondialdehyde (systemic oxidative stress) produced throughout the body was shown to be elevated in plasma from NASH patients when compared to normal subjects and its levels were correlated with insulin-resistance status [29]. The former response involved activation of the c-Jun NH2-terminal kinases (JNK's), endoplasmic reticulum (ER)

stress, unfolded proteins response (UPR), and the ceramide pathway by blocking nuclear factor κB (NF-κB) expression at different levels [29]. In liver tissue, HSP70 downregulates TNF-α and inducible nitric oxide synthase (NOS2), genes that increase the inflammatory response in rodents. In addition, HSP70 in humans induces apoptosis and increases the concentration of cyclopentenone prostaglandins, a potent local inhibitor of inflammation. In human liver and fat tissues, the suppression of HSP70 was strongly correlated with the upregulation of JNK1 and JNK2 [29]. The authors hypothesized that the senescence-like state in fat cells have evolved in obese individuals as an adaptation to the metabolic overutilization of fat cells, supporting the observation that hepatocyte senescence predicts NAFLD progression to NASH and to cirrhosis. Patients with different grades of ESLD from NASH or HCV had significantly decreased levels of glutathione reduced and increased levels of glutathione oxidized in plasma when compared to healthy controls [30]. Therefore, a continuous increase in the cell oxidative stress consumes the antioxidant protective mechanisms and increases the spillage of oxidative molecules. Increased oxidative compounds accumulation may induce a progressive larger number of liver cells into senescence which in turn will enlarge the SASP component worsening the inflammatory environment with further increase of stressors into the liver mieleu by triggering an activation of local and systemic immune-regulators (**Figure 3**).

In old mice, hyperglycemia increased chromatin remodeling and polyploidy levels; changes observed as well in non-obese diabetic mice [31]. Genes involved in glycemic control and metabolism are also involved in inflammation such as Ppargc1a (PGC-1α). It acts on the histone deacetylase SIRT1 as a metabolic sensor in hepatocytes and increases gene activation involved in the gluconeogenesis pathway [31]. Furthermore, PGC-1α also plays a role in lipid metabolism [31]. Through the thyroid receptor pathway, it induces the expression of Srb1 (Scavenger receptor B member 1), enhances the uptake of cholesterol esters from high density lipoproteins (HDL) in the liver and inhibits the expression of Srebp-1 (sterol regulatory element-binding transcription factor-1) down-regulating fatty acid synthesis [31]. As SASP builds up due to an increasing number of cells entering senescence, an increasing insulin resistance state starts to develop with its manifestation, hyperglycemia which further favors replicative cell arrest.

The fat compartment has emerged not only as an energy reservoir but as an endocrine organ capable of modulating metabolic states where the adiponectin/leptin ratio determines an anti or pro-inflammatory response. Leptin is a 167-aminoacid hormone expressed predominantly in adipocytes. Its signaling is an important determinant of food intake, adiposity and energy expenditure [32]. In the *ob*/*ob* mouse, a homozygous mutation in the gene that encodes leptin is associated with increased appetite, obesity and an insulin-resistant state. When leptin was provided to the ob/ob animal, there was a dramatic improvement in glucose homeostasis and energy metabolism. Although leptin related glucose homeostasis is largely conserved in rodents and humans, most subjects with insulin-resistant diabetes have a hyperleptinemic state with a central resistance to leptin [32]. Adiponectin is a protein hormone of 244 amino acids synthesized as a monomer of 28–30 kDa and assembled in various molecular weights: low, medium and high molecular weight (LMW, MMW and HMW) oligomers [33, 34]. HMW oligomers are the major relevant forms in terms of physiological activities of adiponectin while, low amounts of HMW oligomers represent an independent risk factor for several metabolic pathologies such as obesity-related diseases. Adiponectin plays a pivotal role in energy metabolism being an insulin-sensitizing hormone and it is involved in a wide variety of physiological cellular processes including inflammation, immunity and vascular

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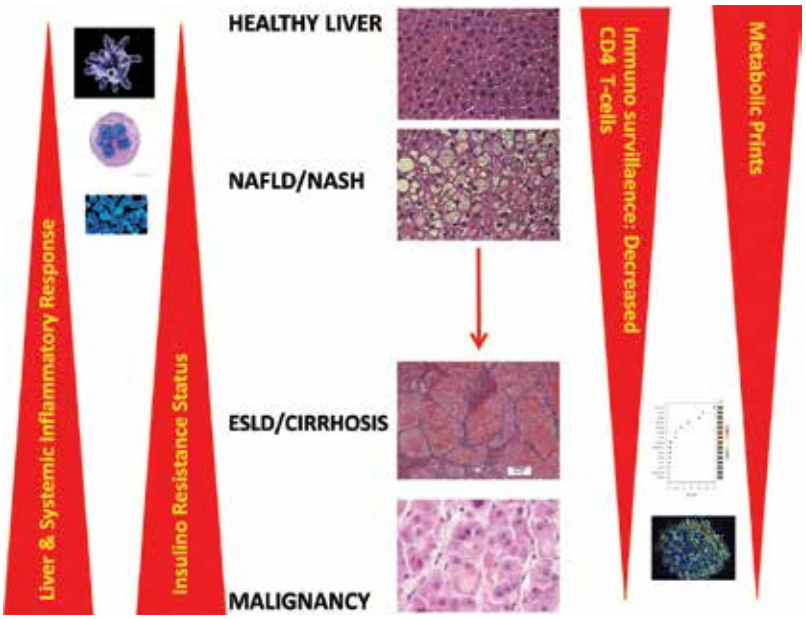

Figure 3. *Local and systemic responses that occur in the progression of NAFLD to NASH and ESLD associated HCC and their metabolomic print.* The liver local progression from NAFLD to NASH is associated with a local inflammatory response that eventually involves several other organs evolving into a systemic reaction. The systemic inflammatory response is associated with the development of an insulin resistance status and the metabolic syndrome phenotype: HTN, central obesity, DMType II and NASH. The continuous liver lipotoxicity decreases mitochondria function and decreases ATP production as well as enhances the secretion of lipid intermediates which are toxic to CD4 T lymphocytes. All together enhances a regenerative stimulus of senescence cells with mitochondrial dysfunction generating at some point a metabolic swap of ATP production to the cytosol, which may be associated with a mitochondrial generated apoptotic switch and in an environment of progressive fibrosis and therefore low oxygen and nutrients delivery favoring the survival of the already highly mutated cells which in turn have escaped physiological cell cycle control and immuno-recognition assuring cell clone growth. Metabolic disturbances precede variations in cell cycle and genetic expression creating metabolic signatures of liver status in health and disease.

physiology. Adiponectin acts through three major physiologically different and distinctly expressed receptors: AdipoR1, AdipoR2 and T-cadherin. Adipo receptors mediate pleiotropic adiponectin actions through signaling mechanisms involving AMPK, ERK1/2, AKT and P38. In addition, Polymerase I and transcription release factor (PTRF) regulates adipocyte differentiation, perhaps fat cell senescence and thus may determine fat compartment expandability, condition that under continuous HFD exposure increase the spill-over of FFA to the liver in combination to a pro-inflammatory adipokine repertoire [35].

2.1.2. Signaling of energy expenditure and metabolism

Rapamycin has an effect in cell life span with significant changes in the liver transcriptome, effects that are more pronounced when animals are exposed to caloric restriction [36, 37]. In a rodent model, rapamycin prevented senescent changes with significant differences by gender but with some common genetic pathways, mainly in the preservation of mitochondrial function. Those pathways included protein ubiquitination, NRF2-mediated oxidative stress response and glucocorticoid and OGF-1 signaling [36, 37]. Cell culture studies, indicated that treatment with rapamycin decreased mitochondrial membrane potential, decreased O_2

consumption, and increased ATP production [36, 37]. Other effects from transcriptome pathways include a decrease in proteasome activity in parallel with an increase in cell autophagy, suggesting protein quality improvement processes and increased resistance to oxidative cell stress effects associated with reduced cell aging.

A highly conserved signaling pathway in all eukaryotic cells is the Target of Rapamycin (TOR), which plays a central role as regulator of cell growth and metabolism. The mammalian TOR complex (mTOR) encompasses two structurally and distinct proteins. While the mTORC1 is associated with anabolic processes such as protein synthesis, lipid synthesis, nutrient uptake and inhibition of catabolic processes including autophagy, mTORC2 is an insensitive to rapamycin regulation protein. mTOR2 becomes activated by a family of kinases like the serum/glucocorticoid kinase (SGK) and protein kinase C (PKC). mTORC1 is upregulated by growth factors, cellular energy status and is inhibited by the macrolide rapamycin [36–38]. Protein synthesis is one of the most energy demanding cell functions; a favorable redox status activates mTORC1 which in turn exercises its actions down-stream through the ribosomal protein 6 kinase (S6K) and the eukaryotic translation initiation factor 4E binding protein (4R-BP) [36–38]. Due to the high demand of ATP, mitochondrial function regulation is of paramount importance on mTOR signaling pathways. Moreover, mitochondrial dysregulation and continuous mTOR activation may play a metabolic central role in the transformation and survival of cancer cells. Mutated cells with malignant potential may shift their bioenergetic state from ATP mitochondrial production to cytosol ATP production through the tricarboxylic acid (TCA) cycle. A connection between mTORC2 and mitochondrial function and cancer appears to be dependent through the HK2 pathway [38]. Nevertheless, recently mTORC2 has been linked to cytoskeleton regulation through the actin remodeling pathway, which has been suggested to have an effect on insulin sensitivity/resistance balance [36, 37]. Whole energy expenditure was affected by the mTORC1 signaling pathway, as demonstrated in the tissue specific knockout mice where a down-regulation of signaling pathways on adipose tissue also impacts on thermogenesis and systemic sensitivity to insulin [38]. In addition, mTORC2 signaling pathway, in the same animal model was a crucial regulator of liver and pancreas metabolism affecting animal growth and insulin homeostasis. mTORC 1 and 2 signaling in the liver affects systemic glucose and insulin homeostasis mainly due to their effects on Akt and hepatic glucose uptake. Interestingly, liver tumors in the tissue specific raptor knockout mice, showed a shift from glucose to glutamine as the main fuel source, making tumor cells glutamine addictive with high expression of mTORC1 and FGF-21. Rapamycin treatment may be beneficial as it may inhibit growth on glutamine addictive tumors. Some liver transplant programs switch their immunosuppression protocol from tacrolimus to rapamycin in patients with high risk for HCC recurrence after transplantation. However and on retrospective studies, its effect on long term overall survival on patients after liver transplantation for HCC, have had conflicting results [39, 40].

The functional relationship between poly-unsaturated lipid metabolism, inflammation and cancer development has been discussed in multiple avenues. Cyclooxygenases (COX's) and lipoxygenases (LOX's) are enzymatic families that metabolize poly-unsaturated fatty acids. COX is present in two isoforms (COX-1 and COX-2) that produce prostaglandins (PG's) and thromboxanes, respectively [41]. LOXs constitute a family of dioxygenases that insert O_2 into poly-unsaturated fatty acids with regional specificity [41]. These metabolites are biologically active hydroperoxyeicosatetraenoic acids that upon reduction forms hydro-eicosatetranoic

acids (HETE's), while the metabolism of linoleic acids preferentially results in hydroxyl-octadecadienoic acids (HODE's), metabolites known to modulate inflammation and carcinogenesis [41]. An excess of poly-unsaturated fatty acids could enhance a higher production of HETE's and/or HODE's with an override of pathways that enhances cancer development. Hepatic COX-2 overexpression induces spontaneous HCC formation in vitro and in mice through Akt, SKT33 and mTOR signaling cascades [42]. In the healthy liver, the inhibitor of the prostaglandin degrading enzyme 15-PGDH potentiates liver regeneration after partial hepatectomy when compared to control and sham animals [43]. Thus, prostaglandin active derivatives have the potential not only to modulate local inflammatory responses but to promote cell regeneration in the healthy cell and potentially reversal of cell arrest in the senescent cell.

2.2. Metabolism in the liver graft

Evidence seems to indicate a peculiar aging pattern for liver grafts after transplantation. Biological age of the graft does not correspond to its behavior when transplanted to a different environment of a younger recipient [44]. One of the most important intracellular protease systems is represented by the proteasome, the central catalytic unit of the ubiquitin-proteasome system (UPS). No difference in the accumulation of oxidized proteins and polyubiquitin conjugates with maintenance of their proteolytic activity was found in liver grafts after transplantation from younger donors to older recipient when compared to liver grafts from older donors placed into younger recipients. Furthermore, there was an increase of the ß5i/α4 ratio, suggesting a shift towards proteasomes containing immune-subunits [44]. Thus, it appears older liver grafts transplanted in younger recipients switched their biological metabolism to resemble the recipient's metabolic age. However, the pattern of liver cell senescence may differ. Liver biopsies, as judged by the senescent markers telomerase and SMP-30 from older transplanted livers showed histological damage in asymptomatic patients with up to 43% and 64% at 5 and 10 years, respectively [45].

In the warm ischemic/reperfusion liver model, glycogen synthase kinase 3 (GSK-3) inhibition ameliorated liver injury upon reperfusion through an energy-dependent mitochondrial mechanism [46]. GSK-3 is a serine/threonine kinase regulated by inactivation through serine phosphorylation. GSK-3 inhibition down regulates the opening of mitochondrial permeability transition pore (MPTP) site, preventing leakage of mitochondrial respiratory chain proteins; a key step in the activation of caspase dependent apoptosis and therefore mitochondrial-dependent cell termination. This effect was present in young animals but abrogated in old animals, and a partial response was re-established in the older group by glucose infusion with hepatic glycogen build up storage [46]. Authors speculated that during reperfusion glycogen degradation provides mitochondrial fuel in forms of glutamate and α-ketoglutarate maintaining enough energy levels that preserve mitochondrial membrane integrity or mitohormesis lowering ROS production, factors needed to decrease MPTP susceptibility. Former approach in the human was entertained, where liver graft glycogen replenishment was performed during the donor phase and evaluated upon reperfusion [47, 48]. Metabolic benefit with improved organ graft function was observed only in borderline grafts and the ones with high fat content. Nevertheless, the concept of metabolic replenishment with further graft function improvement may be refined by strategies of ex-vivo euthermic graft perfusion prior implantation [49–56].

2.3. The chronically diseased liver

Hepatocyte senescence expression has been shown to be present in up to 80% of the cells in advanced liver disease [57]. The effects of insulin in the liver cell are mediated through two main cellular pathways: the phosphatidylinositol 3-kinase (PI3K)-Akt and the Ras-MAP kinase (MAPK) pathways. While both pathways are active in the regulation of cellular growth, proliferation and differentiation the PI3K-Akt mediates the metabolic actions of insulin. Those actions include activation of mTOR1 and its S6 kinase and the inactivation of glycogen synthase kinase-3 (GSK3) as well as its AS160 with nuclear exclusion of the Forkhead box protein (Fox01) [57]. In culture, HepG2 cell lines showed a signaling defect downstream of the Akt pathway with an impact upon insulin mediated Fox01 cytosol sequestration and AS160 phosphorylation; a cascade that translated into insulin resistance of older cells when compared to younger cells. Nevertheless, maintenance of the senescent state requires an active role in the transcriptional activity of Fox01 as cell cycle inhibitor, even in the presence of growth factors. Thus, it appears gluconeogenesis and insulin resistance are unwanted but unavoidable effects of Fox01 gene, which is involved in cell cycle arrest, detoxification of oxygen species, DNA repair and gluconeogenesis [57].

FA overload can damage the respiratory chain in the mitochondrion through a dual role: as an un-coupler and as an inhibitor [13]. Impairment of the key respiratory state 4 → 3 can occur via inhibition of ATP-synthase thereby producing an increase production of ROS irrespective of ADP concentration. The concept of redox-optimized ROS balance (R-ORB) postulates that ROS efflux from the mitochondrion will attain a minimum at intermediate values of oxidation, when VO_2 reaches a maximum following ADP stimulation. Under state 3 respiration, GSH and thioredoxin systems are essential for minimizing ROS release from the mitochondria [13]. Moreover, mitochondria from cells with chronic liver disease under oxidant challenge displayed a two-fold increase in H_2O_2 emission when compared to controls along with a 50% decrease in GSH [13]. Since 90% of GSH in plasma is excreted by the liver, glutathione sp. could serve as a surrogate of cell/mitochondrial stress and their ratio in plasma may reflect overall liver redox balance [24]. In animal models of liver malignancy, with or without cirrhosis glutathione sp. (glutathione reduced-GSH, glutathione oxidized-GSSG and ophthalmate) predicted the growth of malignant cells on normal livers as early as 14 days after malignant cells implantation and differentiated animals with cirrhosis by tumor status (HCC+ vs. HCC−) [58, 59]. Furthermore, glutathione sp. in plasma were part of the metabolic signature that discriminated healthy controls and subjects after liver transplantation with normal graft function from subjects with chronic liver disease (**Figure 3**). In addition, metabolic prints graded patient's degree of end stage liver disease which correlated with the MELD score, and they were able to separate patients with cirrhosis by tumor status, i.e. HCC+ vs. HCC− [30].

Others argued mitochondrial dysfunction by FA's respiratory chain uncoupling is incompatible with thermo-regulatory principles that governs mitochondrial respiratory chain through energy demand: intracellular lipids will accumulate whenever FA's supply exceeds the energy needs of the cell [13]. While TAG-LD in cells from a trained individual increases as the source of energy, in the diabetic obese subject TAG-LD are the result of accumulation with the subsequent potential overproduction of lipid derived toxins in the form of LCFA-CoA, diacylglycerides (DAG) and ceramide, metabolites responsible, at least in part for the development of insulin cell resistance [13]. The former theory is attractive in the heart and skeletal muscle. In contractile

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cells, optimal excitation-contraction coupling requires an optimal energy and O_2 supply which in turn affects the Ca^{2+} handling at the sarcoplasm reticulum (SR) release channels (ryanodine receptors), the SR Ca^{2+} pumps and the sarcolemmal Na^{+}/Ca^{++} exchanger. The heart at rest beats in average 100,000 times per day catalyzing about 6 kg of ATP to ADP. The mitochondrion provides the ATP needed for contraction (≈66%) and the ATP needed for ion transporting (≈33%) essential for the cardiac electrical activity. Thus, the link among lipid supply and mitochondrial function, insulin sensitivity/resistance and ion pump exchange is established for optimal cardiac function or dysfunction in the obese individual [13]. Perhaps, there is no argument lipid oxidation confers a metabolic advantage during starvation and exercise, but its role as the fuel of election during food abundance against metabolic disease deserves further studies.

In liver, cellular senescence is associated with a pro-fibrogenic environment and the relation between advanced liver fibrosis and shortening of the cell telomere appears to be consistent [11, 60]. Telomeres are repetitive DNA sequences (TTAGGG) associated with the specialized protein shelterin. They are located at the chromosomal end acting as a cap that stabilizes and protect the chromosome from erosion and miss-identification as DNA breaks. During normal cell division, telomeres shorten due to the "end replicating problem": the inability of DNA polymerases to fully replicate the 3′ end of chromosomes [61]. Germline cells overcome this problem by expressing telomerase, a reverse transcriptase that maintains telomere length by synthetizing new DNA sequences at the end of the chromosome [60]. The telomerase complex includes a reverse transcriptase (TERT) and the RNA component (TERC) [61]. In other somatic cells, continuous cell division results in telomere shortening which in turn start signaling cell arrest mechanisms, i.e. senescence or apoptosis. Failure of cell arrest signaling, as in a silence p53 state sparks further cell proliferation with chromosomal end-to-end fusions and instability. In addition, exhaustion of liver regenerative paths and invested mechanisms of telomere repair could be overcome under continuous and chronic cell injury with subsequent acceleration of cell senescence and aging. Some studies had shown that telomere biology is involved in HCC initiation and its progression [60]. Therefore, telomere shortening is a physiological marker of cell aging signaling and/or cell arrest preventing further cell division; failure of cell arrest may end in chromosomal instability and subsequent mutations favoring tumor development [11, 61]. In fact, the strength of the DNA damage response (DDR) in the normal cell depends ultimately to the degree of p53 gene regulation: a higher p53 response is associated with apoptosis, a lower response is associated with cell senescence and a silence p53 response may favor tumor development and growth [62]. In addition, a sirtuin (SIRT7) showed an in vivo hyperacetylation of p53 and the SIRT7 knockout mice suffered among other maladies steatotic liver disease. Sirtuins were initially identified in yeast as the Silent Information Regulator (SIR). In mammals, SIRT protein family comprises seven distinct members involved in cellular survival, senescence and tumorigenesis [62]. The SIRT7 knockout mice showed a 2.5 fold increase in the liver triglyceride content and an increased accumulation of hepatocyte inflammatory markers [62]. Findings that were associated with liver cells mitochondrial dysfunction through a deacetylate GABPß1 mitochondrial protein pathway and with the development of HCC through maintaining a deacetylated state of H3K18 at promoters sites of many tumor suppressor genes [62].

Lipodystrophic syndromes are rare and heterogeneous diseases, genetic or acquired, where partial atrophy is associated with a phenotype consistent with insulin-resistant diabetes, dyslipidemia and NAFLD. Although the genetic cause of these syndromes are largely

unknown, most of the monogenic diseases have in common primary alterations in the fat tissue consistent with disturbances of the adipogenesis process or defects in the formation, maintenance and/or regulation of the lipid droplet [63]. Acquired syndromes are seen mainly after HIV therapy with anti-retroviral agents as zidovudine and stavudine (tNRTI's). Agents known to render mitochondrial toxicity with metabolic disturbances similar to the metabolic syndrome seen in obesity. This metabolic adverse effects include premature aging associated with impaired prelamin-A maturation [63]. Lamin-A alterations could produce fragile nuclear envelopes, alter chromatin organization, increase oxidative stress and promote premature senescence at the cellular level. The metabolic disturbances observed in genetic or acquired lipodystrophic syndromes support the hypothesis of a primary fat compartment dysfunction as the source of metabolic disturbances, similar to the ones detected in obesity.

Chronic liver disease is associated with an increased translocation of intestinal bacteria contributory to the liver inflammatory response and may promote the development of HCC [12]. Liposaccharide (LPS) produced by Gram (−) bacteria hosted in intestines from obese humans and rodents was associated with the transition of NAFLD to NASH and consequently to its progression to cirrhosis and HCC. LPS is recognized by the Toll like receptor 4 (TLR4) which is expressed upon cell activation on migrating and local macrophages (Kupffer cells). TLR4 is central for the secretion of TNF-ß and IL-6, cytokines present in the chronic inflammatory environment that precedes the detection of malignancy [12]. Further support to the role of LPS was found by interventions such as gut sterilization, removal of LPS or inactivation of TLR4; maneuvers that diminished tumor growth in chronically injured livers [12]. In experimental models, dietary or genetic obesity alterations on the gut microbiota increased levels of metabolites like deoxycholic acid (DCA) that in turn damages DNA. The enterohepatic circulation may further enhance the concentration of such metabolites by both encouraging the senescent-associated secretory phenotype response and favoring a tumor-promoting environment.

3. Cellular senescence

3.1. Hepatocytes

Historical views of liver cell replication supports the physiological properties of the hepatocytes to restore function as response to parenchymal loss [64]. However, massive or unending injury may overcome regenerative processes or may promote a dysfunctional repair process leading to progressive liver fibrosis, development of portal hypertension and eventually liver failure. Senescent status was induced in HepG2 cells by exposure to H_2O_2. Its consequences and metabolic activity were interrogated [18] and morphological changes were noted with respect to SA-ß-GAL and SAF's expression, cell cycle arrest as well as the upregulation of p53, p21 and p16 genes. Regarding cytokine expression, IL-8 was upregulated while IL-6 was downregulated. Disturbances in glucose and lipid metabolism were evident with upregulation of growth hormone/IGF1 (SOCS2) and glycolysis (PGM2LT). Nonetheless, the downregulation of glucogenolysis and gluconeogenesis (G6PC) were more prominent. The unsaturation of fatty acids was hyperactive (FADS3) with parallel hypo activity of lipoprotein and hepatic lipase activity through the Apo-lipoprotein (APC3) system. APC3 also limits the uptake of chylomicrons by the liver

cell. Other fatty acid downregulated proteins included SORL1 (involved in the uptake of LDL), ACSM2B (a medium-chain fatty-acid-CoA ligase) and PHGDH indirectly involved in amino-acid synthesis [18]. In addition, senescent cells secreted a variety of bioactive molecules including pro-inflammatory cytokines and chemokines that may influence extracellular matrix and the micro-environment but as well modulate the immune response with the promotion of macrophage migration leading to further increase in the inflammatory mielue [65]. Monocyte chemotactic protein (MCP-1) could provide a signal for monocyte recruitment into the liver followed by activation of Kupffer cells with the upregulation of death ligands. The expression of Fas ligand, TNF-α, and TNF-related apoptosis inducing ligand (TRAIL) further aggravates lipo-apoptosis [66]. In addition the FFA palmitate increases the expression of TRAIL and abrogation of the TRAIL receptor expression suppresses the inflammation induced by nutrient excess in mice [66].

Prior assumptions on cellular senescence determined that cell cycle arrest was a mechanism to protect the cell towards tumorigenesis. Nevertheless, it has been shown that the cell in cycle arrest can produce pro-inflammatory mediators, the senescence-associated secretory phenotype that promotes tumor growth [67]. During chronic liver disease, senescent machinery becomes "hijacked" perhaps triggering proliferation and transformation of hepatocytes, thus, promoting metabolic adaptation which may enhance tumor grafting and growth [68, 69]. The above metabolic paths could at least in part, be mediated by the over expression of the phosphatase and tensin homolog (PTEN) described in T-leukemia but later shown in liver tumors to inhibit the pentose phosphatase pathway (PPP) by binding to glucose-6-phophodiesterase (G6PD). With no active G6DP dimer, cells favor glycolysis with the production of lactate even in the presence of oxygen [70].

Aging and senescent liver cells have different genetic paths that may converge to similar metabolic traits. Aging liver cells have a proliferative response after injury associated with the repression of C/EBPα, Farnesoid X Receptor (FXR), telomere reverse transcriptase (TERT), and a decrease in the Wnt signaling pathway [71, 72]. A physiological Wnt signaling pathway involves a soluble ligand that binds to the Frizzled receptor (Fzd) and the LRP5/6 co-receptor on the plasma membrane; this interaction activates the cytoplasmic Disheveled protein which inhibits the ß-catenin (Ctnnb1) destruction complex (APC, GSK3ß, and Axin) by preventing Ctnnb1 phosphorylation and its subsequent destruction. Stable ß-catenin (intact Wnt signaling) translocates to the nucleus to form a complex with Lef and Tcf transcription factors that target genes as c-Myc and Cyclin D1. In cell culture and a mice model of HCC, tumor growth was ablated by the suppression of N-Myc downregulated gene 1 (NDRG1) expression; it promoted HCC cells to go into cell arrest [73]. The induction of senescence on malignant cells was accomplished by upregulation of the tumor suppressor genes p53, p21 and p16 in addition to decreased phosphorylated Rb. Senescent liver cells response to injury included transcription of Nf-kB, Myb, Nkx2-1, Nr5a2 and Ep300 factors; proteins known to be involved in inflammation, cell differentiation, lipid metabolism and chromatin remodeling. In addition, the chronic inflammatory phenotype of senescent cells induces telomere dysfunction and accelerates liver cell aging [74]. Thus, decreased physiological cell signaling that occurs with aging plus stress induced cell senescence may add to the lipid toxic microenvironment by promoting a vicious circle that overrules redundant mechanisms that prevent uncontrolled cell division. Mechanisms that imply an apoptosis "switch" from a pro-apoptotic to an anti-apoptotic status. Nonetheless, it is no clear the role of mitochondrial Bcl-2 proteins family and their expression may determine cellular fate [75].

Cellular events that follows are the activation/repression of factors involved in cell proliferation. In the liver cell, the known transcriptional shift includes activation of FOXO3, FOXII, E2F1, c-jun, C/EBPß, Myb, USF and neutralization of inhibitors of cell proliferation such as Rb family and C/EBP family of proteins [76]. In C/EBP-S193A mice, failure to stop liver regeneration after surgery correlated with the epigenetic repression of C/EBPß, p53, FXR, SIRT1, PGC1α and TERT. The repression was performed by a protein formed by C/EBPß-HDCAC1 complex which also inhibit the promoters of enzymes for glucose synthesis PEPCK and G6P [76]. The response of cell cycle engaged hepatocytes and cell cycle arrested hepatocytes (senescent cell) to injury is different and it may awake an unregulated cell growth on quiescent stem liver cells [76, 77]. Oval shaped liver cells may differentiate into cholangiocytes with a distinct metabolism and perhaps pathway towards malignancy [15, 16]. Although cholangiocytes are metabolically very active cells involved in the secretion and resorption of water and soluble bile components, they are not directly involved in the metabolism and/or regulation of biliary lipid species (cholesterol, bile acids and phosphatidyl-choline vesicles) [78, 79].

3.2. Hepatic stellate cells (HSC) & portal myofibroblasts (MF)

HSC are quiescent cells that express typical markers of both neural cells and adipocytes (glial fibrillary acid protein-GFAP, peroxisome proliferator-activated receptor gamma-PPAΫ, and adiponectin receptors). They are activated by cytokines, growth factors, ROS, damaged cells and apoptotic bodies [64]. In health, MF are located adjacent to bile duct epithelia and are the first responder to biliary injuries. Upon activation HSC's acquire a MF phenotype, cells that upon phagocytosis of LD and/or apoptotic bodies from damaged cells get additional energy and became Fas-ligand and TNF-α unresponsive to apoptosis; mechanism in use for increase collagen synthesis and deposition [64]. Furthermore, activation of the adenosine receptor A_{2A} increases HSC proliferation and inhibits death and senescence by down regulation of p53 and Rb through the cAMP-PKA/Rac1/p38 MAPK pathway [80]. Activated MF's express CCN1/CYR61, an important regulator of inflammation and wound healing. Cystein-rich 61-protein (CCN1/CYR61) is a matrix-cellular protein that induces senescence at later stages of wound healing by promoting tissue remodeling through fibrogenic cell apoptosis and attenuation of TGF-ß signaling [81]. HSC and MF senescent fibrogenic cells no longer proliferate, thereby reducing the load of ECM deposition. In addition, senescent fibrogenic cells express an increase in the secretion of metalloproteinases (MTP's) leading to matrix degradation. Apoptotic fragments from HSC and MF are cleared by natural killer cells promoting wound healing, the best characterized mechanism of fibrogenesis resolution [64, 81]. NF-κB is a key regulator for HSC survival and proliferation by maintaining the expression of Mcl-2. Inhibition of NF-κB increases HSC apoptosis by up-regulation of the JNK pathway. Thus, the activation as well as the induction of senescence/apoptosis of HSC/MF are normal wound healing mechanisms that promote the establishment of normal organ architecture and function with clear paths of initiation and resolution.

During chronic cell injury, such as in a state of high caloric intake enriched with lipids, an increase and progressive pool of biologically active HSC's may become prominent [11]. An incremental chronic state of fibrogenesis alters hepatic architecture leading to a concomitant increase in portal flow resistance, portal hypertension and the development of collateral circulation. In addition, HSC's produce a microenvironment with altered extracellular matrix (ECM) that provides biochemical and mechanical cues to the growth and establishment of tumor cells [67]. Nevertheless,

since 90% of the HCC's flourish in a highly progressive fibrotic ECM, the question raises if it is the changes on the microenvironment that further promotes metabolic transformation with an "apoptotic switch" and tumor development. Interestingly, progressive liver fibrogenic ECM becomes enriched with vascular growth factor (VGF) receptor promoting angiogenesis, paving the way for the much needed arterial high O_2 supply for HCC expansion [67].

The different components of the ECM, cellular and non-cellular interact directly and indirectly with malignant cells therefore changing the phenotype of the evolving cells that in turn produces feedback signals to further adapt the microenvironment to the needs of the malignant cell. The link between the actin cytoskeleton and the microenvironment provides an input of intracellular contractile forces capable of regulating signaling pathways fundamental to the definition of cell phenotype, mechanism that constitute the ECM "out-side-in" code to the cell. In response, the anchored cells expressed adhesions molecules and secreted proteins that signals HSC and other ECM regulators increasing anchoring sites in response to the "in-side-out" signaling [67]. Therefore, the metabolic transformation of the already stressed parenchymal cells help to choose a path different to senescence and necrosis but to a path of unregulated regeneration, thus escaping apoptosis. A path that needs an ECM differentiation to assure cell survival in a non-efficient energy redox status.

3.3. Sinusoidal endothelial cells (SEC)

SEC's are specialized endothelial cells that lie flat in the liver sinusoids along and in direct contact with the hepatocytes. Through their membranes and specialized pores or fenestra passes high concentrations of metabolites, proteins and other blood compounds, traffic which is regulated by the size of the fenestra. SEC's play a critical role in immune-activation, rolling of T cells, macrophages and PMN migration. Liver sinusoidal endothelial cells may be affected with age and obesity. SEC from old individuals have impaired and reduced expression of VEGF likely due to impaired nuclear transport of P-STST3 and P-CREB transcription factors [82, 83]. In a rodent model of sepsis, endothelial nitrogen oxide synthase (eNOS) deficient mice and aging mice had the same mortality and mitochondrial dysfunction upon the isolation of SEC mitochondrion [84]. In obesity and during early fibrogenesis, SEC lose their fenestra, decreasing the exchange of metabolites and increase the secretion of several basement membrane components (type IV collagen, perlecan, entactin and laminin) [64]. Authors concluded that an endothelial base-line dysfunction in the aging animal is manifested by a weakened antioxidant response and inappropriate energy production from mitochondrial dysfunction due to a tipped-balance of the SEC oxi-redox systems when exposed to additional stress. This is seen in the obese towards a state of energy depletion and cellular death, apoptosis or activation of a pro-coagulant/pro-fibrogenic phase. The changes of SEC's with aging may limit O_2 delivery and availability to liver cells with its potential effects on mitochondrial function, a pro-fibrogenesis state and the promotion of insulin resistance status. Changes exaggerated in obesity, implying obesity may promote accelerated SEC aging processes. Interestingly, endothelial cellular senescence was inhibited in vitro and in the rodent by the activation of the liver x receptor (LXR), a nuclear receptor involved in the control of hepatic lipid and cholesterol metabolism [85]. Furthermore, LXR has been shown to play an important role in glucose metabolism, cytokine production and anti-inflammatory response.

Three types of SEC's co-exist in the normal liver sinusoid: mature SEC, SEC progenitors and bone marrow-derived SEC progenitors [86]. Mature SEC are gatekeepers of fibrogenesis by maintaining HSC in their inactivated state. SEC's regulate sinusoidal blood flow through their action on HSC and thus keep a low portal pressure [86]. In addition, mature SEC's have the largest endocytic capacity in the body fulfilling their dual cell clearance capacity (from the arterial/systemic and portal/gut systems). The liver endocytic function has been implicated in a liver-renal axis where the lack of SEC-stabilin-2 receptors inhibit the clearance of toxic molecules that manifest with mild liver fibrosis without liver dysfunction but with renal glomerular fibrosis. Not only do SEC's have many glycoproteins that serve as receptors for bacterial epitopes but as receptors for immune-modulation and pro-coagulant activity. The above mentioned SEC functions are at least partially lost at the time of sinusoid capillarization [86]. SEC capillarization is characterized by the disappearance of the fenestrae, development of a basement membrane and the appearance of characteristic markers. This phenomenon happens in chronic liver injury and it precedes activation of HSC and sequestration of macrophages. The angiogenesis process that follows is mediated by VEGF, an angiocrine response that drives neo-vessel formation in direct proportion to the degree of the sinusoidal pressure gradient. Furthermore, SEC pseudo-capillarization refers to changes that occur in endothelial cells associated with aging and senescence. It is manifested by a decrease of up to 50% of their fenestrae, development of a patchy basement membrane and partial SEC dysfunction [86]. Chronic exposure of high fat diet may accelerate aging/senescence of SEC, endothelial dysfunction with recruitment of systemic immune cells and activation of Kupffer cells inducing HSC into a fibrogenic state followed by an angiocrine response that decreases hepatic blood flow, O_2 delivery, and clearance of toxic molecules. As metabolic stress of neighbor hepatic cells already in mitochondrial distress due to fat accumulation progresses, a constant and growing inflammatory mielue enhances tumor development, immune-recognition failure and malignant cell expansion.

Interestingly, aging endothelial cells from the fat compartment of mice was associated with adipose dysfunction manifested by ectopic (liver) fat deposition and adipose tissue fibrosis, increased adipose mitochondrial oxygen flux, altered lipid utilization, increased tissue oxidative stress and lower gene expression in visceral fat [87]. Nevertheless, and most important, these findings were associated with reduce fat tissue vascularity, reduced angiogenic capacity and endothelial dependent dilation with reduced nitric oxide (NO) bioavailability [87]. Limited oxygen mitochondrial availability contributes to the pro-oxidative older adipose tissue phenotype that can further impair both insulin action and vascular function, a key element in local and systemic insulin-resistant related metabolic syndromes. Changes that are exaggerated in obesity, implying obesity may promote accelerated aging processes in many organs.

3.4. Resident liver immunocells

The anatomical location of the liver and its dual blood supply ensures an optimal exposure of antigens to the hepatic resident immune cells not only from nutrients and GI microbiota but from systemic compartments, such as the adipose compartment. Kupffer cells in concert with NK, CD4+ T-cells, and local antigen presenting cells modulates the liver immune status. Kupffer cells constitutes 80% of the tissue fixed macrophages and 20% of the non-parenchymal cell population of a normal liver [9]. Their characteristic macrophage activity is polarized mainly in portal tracts where the antigen dynamics is higher from food and bacteria. Innate macrophages have the potential to initiate an inflammatory response of different proportions by upregulating

adhesion molecules such as ICAM-1, and cytokines as TNF-α, IL-1, IL-6, MIP1α, TGF-ß and RANTES. Activation that can only lead to antigen presenting, cell to cell communication and amplification and enrichment of the microenvironment with ROS promoting subsequent parenchymal cell apoptosis/necrosis. Natural Killer (NK) and CD8+ T cells developed a specific signature in livers with NASH from mice under HFD [88]. The depletion of CD8+ T cells protected murine from NASH progression but not from weight gain. In addition, NK T-cells in the liver expresses markers that recognize lipid antigen CD1d [9]. Liver NK cells undergo Thymus clonal double deletion but are positive for CD3 and CD56 and they were thought to be CD1d independent. Nevertheless, hepatic antigen-presenting cells may introduce microbial glycolipid antigens to NK cells, stimulating secretion of Th1 or Th2 cytokines which subsequently initiates an adaptive response. Hepatic NK cells have as well the ability to secrete osteopontin and sonic hedgehog, molecules known to promote the transition from NAFLD to NASH [9].

The most accepted hypothesis, continuous cell parenchymal damage and necrosis adds to a chronic inflammatory environment a dysregulation of the cell cycle regenerative process rendering tandem mutations and thus malignant cells was challenged [89]. On the NEMO knockout mouse, authors were able to develop HCC through a death receptor-independent FADD signaling pathway. Nevertheless, it wasn't until recently that the link between a metabolic hostile microenvironment, immune-recognition failure and HCC presence was established [90]. The enrichment of linoleic acid in the cirrhotic microenvironment of NASH patients promotes disruption of mitochondrial function in a greater proportion than other fatty acids as palmitic acid. Since CD4(+) T lymphocytes have a larger mitochondrial load than CD8(+) T lymphocytes, they not only generate more mitochondrial derived ROS but CD4(+) cells may undergo larger selective loss of mitochondrial function and viability. Therefore, disruption of mitochondrial function by linoleic acid mediates selective loss of intrahepatic CD4(+) T lymphocytes, status associated with HCC presence. Local metabolic changes could alter the immune response to a one that favors malignant cell expansion.

In the obesogenic environment, aberrant activation of immune cells has emerged as key features of the metabolic syndrome. The interaction between the adipose compartment and the liver tissue has been hypothesized as a critical interface for nutrient sensing and metabolic control [9]. In the rodent model, neutrophils infiltrate the adipose compartment as early as 3 days after starting a high fat diet, however its role as well as the role of basophils and eosinophil cells has not yet been clarified. Mast cells, which has been observed in increasing number have been implicated in the secretion of IL-6 and IFN-ϫ [9]. Moreover, leptin, a hormone secreted specifically by adipocytes has been found to be increased during high fat diets and upregulated the expression of leptin receptors on NK T-cells. This regulation is time sensitive, and chronic leptin stimulation change NK cells from an inflammatory like response to a damped one, favoring at long term, in the liver and perhaps in other organs a susceptibility to low recognition of no self-cells, impaired anti-tumor surveillance and a flourishing nest of cancer. The former hypothesis finds support in the obese mice, where it was observed a switch from the normal Th1 immunoresponse to the Th17 immunorepertoire, phenotype that deteriorates autoimmunity [9].

3.5. Extracellular matrix (ECM)

The extracellular matrix (ECM) is formed by a non-cellular component in tissues and organs composed primarily of water, proteins and proteoglycans. Components created an intricate

scaffold where organ cells get structural support with a dynamic and continuous traffic of water, ions, metabolites, proteins and cells on passant to maintain organ physiology. As such, ECM interactions with organ cell components regulate cell differentiation, adhesion, proliferation, migration and survival [64]. The collagen family is the major fibrillar proteins of the ECM and the body (approx. 30% of the total protein contain) [64]. There are three main classes of collagen, fibril-forming which include types I, II, III, XI, XXIV and XXVII the most common varieties and their role is mainly mechanical by conferring tensile strength to both tissue and organs. Fibril-associated collagens with interrupted triple helix (FACIT's) includes type IX, XII, XIV, XIX, XX, XXI and XXII; this subclass of proteins do not form fibrils themselves but bind to the surface of pre-existing collagen favoring fibril enlargement. Finally, type III collagen serves as anchoring collagen between the epithelial cells and the lamina reticularis constituting the basement membrane where type IV collagen is most abundant. Non-collagenous proteins include fibronectin, tenascin, laminins, fibrillins and matrix-cellular proteins. While the former peptides play a major role in cell differentiation, cell growth, adhesion and migration, matrix-cellular proteins, i.e. thrombospondin-1 and 2, osteonectin, osteopontin and cyr-61/connective tissue growth factor (CTGF) serve mainly as a vehicle for cell signaling. Proteoglycans are carbohydrate enriched proteins which retain large quantities of water regulating the smooth trafficking of molecules to and from the cell with numerous signaling active sites for growth factors.

The ECM continuous remodeling is a complex process that integrates proteins and cellular components from local and distal environments [64]. The degradation of ECM proteins are closely controlled by matrix metalloproteinases (MMP's), a superfamily of zinc-dependent endopeptidases highly regulated by specific inhibitors such as the tissue inhibitor of metalloproteinases (TIMP's). In the liver, cellular component involved in collagen synthesis and deposition included HSC, MF and vascular smooth muscle cells [64]. In chronic liver injury, an override mechanism of collagen deposition regulation promotes massive ECM expansion. The characteristic features of abnormal liver fibrogenesis as a consequence of continuous liver injury and activation of collagen secreting cells include damage to the epithelial/endothelial barrier, recruitment of inflammatory cells, secretion of cytokines and other inflammatory mediators, further generation of ROS, progressive deposition of collagen with expansion of ECM and worsening organ fibrosis and subsequent metabolic changes of portal hypertension.

4. Mitochondrial senescence

The mitochondria, a double membrane cell organelle varies in number and its presence is linearly associated with the metabolic activity of the organ and its required energy requirements in form of ATP. Within the mitochondrial matrix a series of biochemical reactions occur. Acetyl-choline primer is reduced through the tricarboxylic acid cycle converting glycolysis-derived pyruvate into NADH and succinate. The former compounds couple another set of reactions at the inner membrane border where the electron transport chain (ETC) is present to boil an oxidative phosphorylation process. The ETC is composed of five enzymatic complexes (I to V; NADH-CoQ, succinate-CoQ, CoQ-cytochrome reductases, cytochrome c oxidase and ATP synthase, respectively) where NADH is the substrate of ETC-C1 and succinate the substrate of ETC-CII [10]. After oxidation, electrons are transferred from Complex I to CII to CIII and finally to Complex

IV where oxygen is reduced to form H_2O. The electron transport process is coupled to a proton pumping process creating a proton gradient between the mitochondrial membranes, gradient that is dissipated by Complex V (ATP synthase) through ATP synthesis. A control mechanism is created by the "proton leak", mechanism that generates heat instead of ATP [10]. Much of the leak is a catalytic reaction generated by the uncoupling proteins (UCP's) which play an important role in reducing proton gradient, heat and ROS [10]. Mitochondrial aging and senescence are linked to reduced ATP production and increase ROS production, i.e. superoxide (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) which are mostly produced because electron leakage at the level of CI and CIII [17]. Mitochondria function benefit from the role of estrogen in plasma through its binding to the nuclear estrogen receptor that enhances a signaling to prevent oxidant stress and also inhibits the renin-angiotensin-aldosterone system [10]. Thus, sex differences in mitochondrial function may explain the disparity in overall survival between men and women, differences that may be taken into account during animal models studies.

The reasons why the mitochondrion conserves a cell independent genome are not clear, but it is intuitive to imply self-energy regulatory processes are united through a fine tune mechanism between energy expenditure (ATP use) and energy production (ATP synthesis) at every organelle level. It may provide an overall advantage for survival of the cell, the organ and entire biological living system. The gradual ROS response theory of aging argues a protective role of ROS in early life, when cell oxidative damage and ROS production are low; however, later in life ROS reaches a level where its beneficial effects (as the one observed in dietary restriction and/or exercise) are overcome by its detrimental effects elicited by a higher cell oxidative stress (as the one observed in high fat diet and sedentary habits) [17]. The effects that are amplified include loss of genomic controls (p53), microRNA dysregulation, loss of function of telomerase reverse transcriptase (TERT) and a lower immune-surveillance status. Although the role of p53 in the mitochondrion is not completely clear, p53 binds to the Peroxisome proliferator-activated receptor Gamma-Coactivator 1 alpha and Beta (PGC-1α and ß) fomenting their inhibition of expression and therefore downregulated oxidative function. In addition, p53 target p16 and p21, factors that triggers G1-phase cycle arrest by inhibiting cell cycle regulatory kinases Cdk4 and Cdk2 [17]. The third known effect of p53 at the mitochondrion level is to promote cell apoptosis by increasing mitochondrial membrane permeability with leakage of cytochrome proteins, a direct activator of the caspase cascade. The function of TERT is highly affected by levels of ROS production and its protective patterns are only observed with low ROS levels. The role of microRNA in the mitochondrial environment remains to be elucidated.

The Mitochondrial Free Radicals Theory of Aging (MFRTA) has been the most popular theory to explain the cell aging process where increasing production of mitochondrial ROS with lower ATP production are the main factors responsible for cell aging and corresponding mitochondrial ultrastructure changes [17, 91]. As mentioned, leakage of electrons at the level of CI and CIII transfer are larger with age and the higher potential for DNA damage. 8-oxo-7.8-dihydro-2'deoxyguanosine (8-oxodG) is one of the most abundant DNA mutations caused by oxidative conversion to guanosine. Furthermore, its accumulation follows an inverse and exponential curve against life expectancy in several mammals [17]. Recently, it was described that humans with longer longevity have a higher content of mitochondrial DNA (mtDNA) per cell in different organs, and support the notion of ethnic background on mtDNA influence and life span. The frequency of mtDNA mutations occurs at different rate depending on the organ. Skeletal and

cardiac muscles, liver and kidney are more affected by somatic mtDNA mutations compared to other organs such as the skin and lung [17]. Furthermore, the clonal expansion of mtDNA mutations occurs via a phenomenon called genetic drift, a random propagation and expansion of DNA mutations occurring at each DNA replication. The drift of mutations may be more important in metabolically more active organs that require more energy expenditure and therefore more ATP synthesis. The expansion of mtDNA mutations may be enhanced not only by its duplication and drift but also by a lower state of DNA damage repair mechanisms [17]. The Base Excision Repair (BER) process is impaired in senescence and aging due to a loss of function to BER associated proteins CSA and CSB. Thus, the increase production of ROS creates a vicious loop of mtDNA mutations than in turn favor an increase production of ROS perpetuating and enhanced organelle dysfunction by defective reparative mechanisms. A naturally occurring thymidine to cytidine mutation in the mitochondrial stressors $tRNA^{ILE}$ gene is associated with phenotypes of hypertension, hypercholesterolemia and hypomagnesemia [10]. Furthermore, the DNA A3243G mutation causes impaired insulin secretion and polymorphisms in the promoter of the UCP2 protein, alterations associated with increased incidence of obesity, reduced insulin secretion and DMII [10].

Mitochondrial function may be impaired in chronic high fat diet challenge as a result of a decrease in ß-lipid oxidation. Indirect evidence showed an accumulation of diacylglycerol and fatty-Acyl-CoA which in turn activates stress-related serine/threonine kinase activity and inhibit glucose transport [10]. Oxidative stress contributes further to impaired insulin signaling increasing UCP2 activity which in turn enhances "proton leak" with uncoupling of the glucose metabolism pathway and decreased ATP production. A progressive higher lipid peroxidation may favor further oxidative stress with DNA damage and low DNA repair by affecting members of the Bcl-2 family, triggering an influx of Ca^{2+} with subsequent opening of the mitochondrial permeability transition pore, cytochrome-c leakage to the cytosol and activation of the caspace-3 complex. Cell self-digestion and nuclear DNA fragmentation overcomes with the typical cell fragments morphology [10]. Alternatively, DNA damage and telomerase shortening results in mutations that may affect mitochondrial function to a level of organelle survival but inefficient ATP production assuring the "apoptosis switch" and diverting biochemical reactions to a cytosolic site for ATP production. The later assumption may find some support in the observations that tumor development and early growth is favored in low O_2 delivery zones and that tumor development is associate with increase lactate production, the Warburg effect [30, 69].

5. Future directions

Prevention of metabolic syndrome and its health consequences is primordial. A healthy diet that is balanced not only in calories but also in its components, specially fats and carbohydrates would avoid fat storage spillage from a saturated fat body compartment. In addition, a substantial use of lean mass through directed exercise will decrease further cell, organ and body aging. In the brain, the melacortin forms a network of neural food sensing connecting signals of metabolic rate with neurological sites that regulates food intake behaviors and energy expenditure homeostasis. Central administration of α-MSH reduces food intake and may also increase energy expenditure resulting in weight loss [92]. Metabolic disturbances in the liver renders liver cell changes that progress from NAFLD to NASH to cirrhosis and malignancy. A non-invasive

plasma based monitoring of such changes on disease progression and treatment response as well as for tumor screening may be possible by metabolomic liver prints in the near future [30].

There have been a myriad or reports on compounds that not only prevent but reverse cell aging and some even malignant development in the animal model [25, 93–102]. Curcumin, the major bioactive compound of turmeric spice, through its antioxidant and anti-inflammatory properties has been claimed to retard tumorigenesis and diabetes and to modulate lipid metabolism [103]. Furthermore, curcumin prevents the development of atherosclerosis and NASH, perhaps by the upregulation of a fatty acid binding protein present in adipocytes (aP2) but also found in macrophages (FABP-4). This protein is a cytosolic protein present in adipocytes and macrophages which modulates the trafficking of lipids/cholesterol processes and activation of inflammatory mechanisms through CD36 upregulation and reduced expression of NF-kß thus, decreasing cytokine secretion [103]. Prior studies showed that high fat diet and obesity promoted liver tumorigenesis by inducing chronic inflammation through the IL6/STAT3 pathway and, STAT3 activated tumors has been showed to be more aggressive in humans. Lycopene attenuated HCC occurrences in the animal model through downregulation of the STAT3 signaling [95]. The aqueous extract of *Ligustrum lucidum* fruit induced apoptosis through the activation of the caspase cascade and cellular senescence by upregulation of p21 and downregulation of RB phosphorylation [102].

Other molecules with promising cell aging and tumor repression properties included the COX-2 and a Na/K/ATP signaling mechanisms. Inhibition of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a prostaglandin-degrading enzyme, potentiates tissue regeneration in multiple organs in mice [43]. During a chemical screen, a low molecular compound was identified capable of selectively inhibiting 15-PGDH with the subsequent increase of PGE2 levels in bone marrow and other organs, accelerating hematopoietic recovery in mice receiving bone marrow transplant and tissue regeneration in the colon and liver. It also promoted tissue regeneration in mouse models of colon and liver injury. Selective COX-2 products may have rescued telomere dysfunction, cell senescence and tissue regenerative potential [74]. However, its mechanism and signal transduction remains to be determined. pNaKtide is a synthetic peptide that conserves the active sequence for the ligand-binding capacity to the ß-subunit of the transmembrane Na/K-ATPase. Although the Na/K-ATPase mainly exercise its function as an ion exchanger pump vital for cell survival, recently it was shown to elicit nuclear signaling that regulates mitochondrial function and cell energy production through a Src/ERK pathway [104–112]. Furthermore, pNaKtide prevents the development of atherosclerosis and fatty liver disease in the HFD mice model with significant amelioration of ROS. In addition, it downregulates collagen synthesis and inhibit growth of human cancer cells in vitro. Translation of promising compounds to the treatment of patients with NAFLD/NASH is expected in the near future to further prevent the consequences of advanced liver fibrosis and HCC development.

Abbreviations

4R-BP	Factor 4E binding protein
ADP	Adenosine diphosphate
AGPAT	sn-1-acyl-glycerol-3-phosphatase acyltransferase

ATP	Adenosine triphosphate
CACT	Carnitine-acylcarnitine transferase
DBC-1	Delete in Breast Cancer-1
DGAT	sn-1,2-diacylglycerol acyltransferase
DM	Diabetes mellitus
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FA	Fatty acids
Fox01	Fork head box protein
GBD	Global Burden of Disease
GPAT	Glycerol-3-phosphatase acetyltransferases
GSH	Reduced glutathione
GSK-3	Glycogen synthase kinase 3
HBV	Hepatitis B virus infection
HCV	Hepatitis C virus infection
HSC	Hepatic stellate cells
HSP70	Heat shock proteins
HTN	Hypertension
IL-1ß	Interleukin-1 beta
IL-6	Interleukin-6
INF-α	Interferon alpha
JNK's	c-Jan NH2-terminal kinases
LD	Lipid droplet
MAPK	Ras-MAP kinase
MF	Portal myofibroblast
MMP	Matrix metalloproteinases
MPTP	Mitochondrial permeability transition pore
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic fatty steatohepatitis
NF-κB	Nuclear factor κB
NO	Nitric oxide
PAP	Phosphatidic acid phosphatase

PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
ROS	Radical oxygen species
SA-ß-GAL	ß-galactosidase
SAH	Senescence associated heterochromatic foci
SASP	Senescence associated secretary phenotype
SEC	Sinusoidal endothelial cells
SGK	Serum/glucocorticoid kinase
SMase	Neural Smase
TAG	Triacylglycerol
TCA	Tricarboxylic cycle
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumor necrosis alpha
TOR	Target of Rapamycin
UPR	Unfolded proteins response

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Chapter 5

The Diagnostic and Prognostic Potential of MicroRNAs for Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (also termed hepatocarcinoma) is the third cancer-related cause of death worldwide. To our knowledge, markers such as α-fetoprotein display poor performance in the early diagnosis and prognosis prediction of hepatocarcinoma. MicroRNAs are an evolutionarily conserved class of small noncoding single-stranded RNA typically consisting of 18–24 nucleotides. They have been reported to act as tumor suppressors or oncogenes via reversely regulating gene expression. Recent evidence has revealed that microRNAs, especially in body fluids such as the blood and urine, display important diagnostic and prognostic potential for hepatocarcinoma. Here, we reviewed currently available data on microRNAs and hepatocarcinoma, with emphasis on the biogenesis and function of microRNAs and their potential diagnostic and prognostic value for hepatocarcinoma. We also discussed the clinical utility perspectives of microRNAs in hepatocarcinoma and possible challenges.

Keywords: hepatocarcinoma, microRNA, diagnosis, prognosis

1. Introduction

Hepatocarcinoma, also termed as hepatocellular carcinoma (HCC) or liver carcinoma, is the most common primary liver malignant disease in adults [1]. One of the most striking features of this malignant tumor is the wide variation in its incidence in different parts of the world. In areas of high incidence, such as China, hepatocarcinoma is among the leading cause of cancer-correlated deaths in recent years, with an annual incidence of approximately 40 per 100,000 [2, 3]. However, the countries in the low incidence, such as the USA, have only 2.3% of cancer-related deaths in

past decades. Globally, hepatocarcinoma is the fifth most common cancer among males and the eighth most common among females [1]. Furthermore, the incidence of this tumor generally increases with age, although there are geographic and gender differences. The precise reasons for this difference is not known, but growing evidence has exhibited that multiple factors including chronic viral hepatitis B (HBV) and C (HCV), aflatoxin (such as aflatoxin B1) exposure, hepatic cirrhosis, obesity, diabetes, and vitamin D deficiency play an important role [4–6]. Although the molecular mechanism of hepatocarcinoma has been unclear, these hepatocarcinoma patients with early diagnosis often have good prognosis with more than 50% of five overall survival rate [6]. This is mainly because they benefit from the curative treatment such as curative resection and orthotropic liver transplantation [6]. However, if patients are lately diagnosed, the cumulative 5-year survival rate remarkably reduces to less than 10%, and tumor recurrence risk noticeably increases (about 70–80% of 5-year recurrence rate). Thus, it is very urgent to identify specific and sensitive markers for early diagnosing hepatocarcinoma at a curative stage, monitoring recurrence of tumor, and predicting prognosis of tumor [6].

Currently, the early diagnosis of hepatocarcinoma is based on the following two classes of methods: imaging examination which mainly consists of ultrasonography, magnetic resonance imaging, and computed tomography and serological tests such as serous α-fetoprotein (AFP) [4, 7]. Although advances in imaging technologies have significantly improved the early screening of hepatocarcinoma, these methods are so costly and unsatisfactory in early diagnosis that is not suitable for daily clinical practice [4, 7]. About serological methods, AFP is the most widely utilized marker for the diagnosis and prognosis prediction of hepatocarcinoma. However, this biomarker is limited because of its modest accuracy (with sensitivity of 40–65% and specificity of 87–96%) and about 30–40% of the false-negative rate for patients with early-stage hepatocarcinoma [8]. Additionally, serum AFP levels of some benign hepatic lesions, such as liver nodular hyperplasia, inflammation lesions of liver, and liver fibrotic cirrhosis, may give false-positive results [8]. Therefore, the reliability of this biomarker to determine hepatocarcinoma is inadequate because of its low sensitivity and specificity.

Emerging evidence has exhibited a correlation between dysregulation of microRNAs and development of hepatocarcinoma. Particularly, microRNAs are characterized by high stability in body fluids (including the blood and urine) and tissue specific in expression patterns, indicative of microRNAs in body fluids acting as potentially novel and ideal biomarkers for hepatocarcinoma diagnosis and prognosis prediction [8–16].

This review attempts to briefly review currently available data on microRNAs and hepatocarcinoma, with emphasis on (1) the biogenesis and function of microRNA, (2) potential diagnostic and prognostic value for hepatocarcinoma, and (3) the different value for hepatocarcinoma induced by different causes. Additionally, we summarized the clinical applicative perspectives and potential challenges of microRNAs in hepatocarcinoma.

2. MicroRNA biogenesis and function

Previous several reports have thoroughly reviewed biogenesis and function of microRNAs [8, 11, 17–29]. In brief, microRNAs are an evolutionarily conserved class of small noncoding

single-stranded RNA typically consisting of 18–24 nucleotides. Originally, they are first transcribed by the RNA polymerase enzyme II into a kind of primary production named as primary microRNA that is characterized by long nucleotide sequences, 5′-cap structure, and 3′-poly-A tail, resembling protein-coding mRNAs. Then, primary microRNAs form a hairpin-shaped stem-loop structure and are processed into microRNA precursors (usually containing 60–70 nucleotides) by the microprocessor complex (consisting of DGCR8/Pasha and Drosha). After that, their precursors are transported to the cytoplasm and treated into a short double-strand duplex structure by another RNase endonuclease III (also called Dicer). Finally, the duplex structure (also called microRNA-microRNA*) is unwound into mature microRNAs by helicases. To date, it has been identified that there are more than 1800 microRNAs in the mammalian genome (miRDatabase) (**Figure 1**) [30]. Functionally, microRNAs are involved in regulating the expression of their

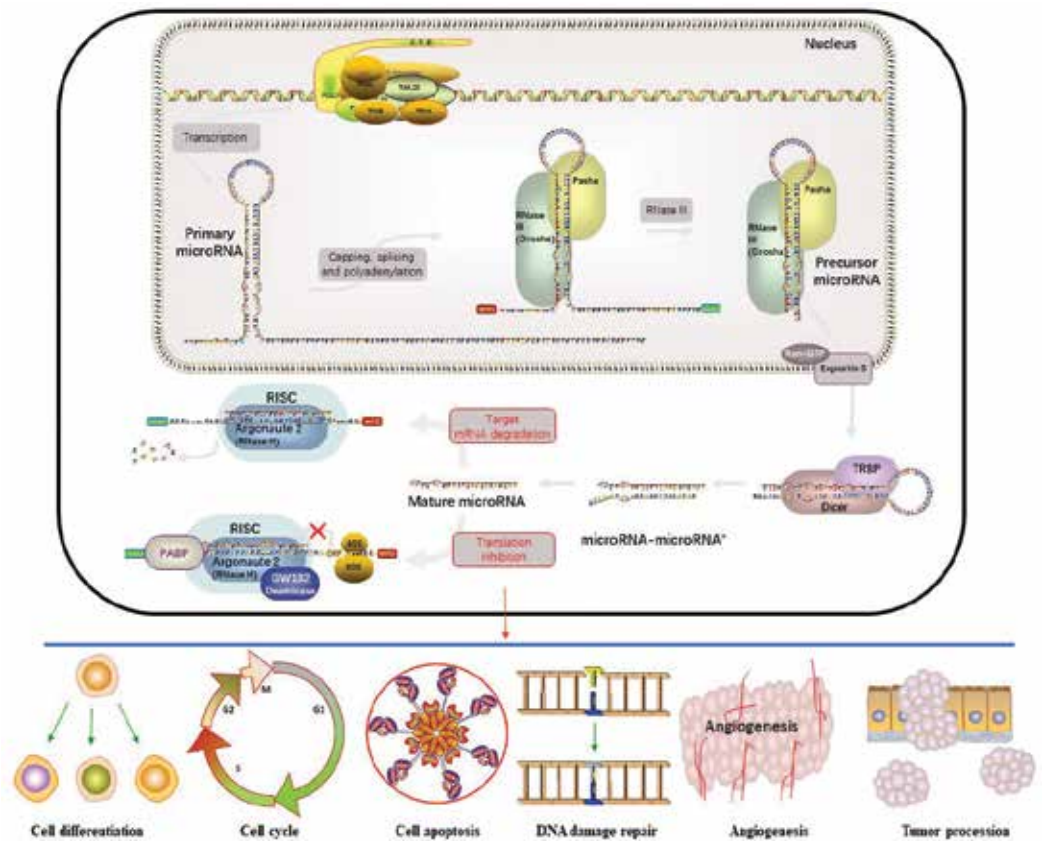

Figure 1. Biosynthesis and functions of microRNA. In the nucleus, the microRNA genes are transcribed into primary microRNAs by RNA polymerase II (Pol II). The primary microRNAs are then cleaved by Drosha and DGCR8 and produce their precursor molecules (also named as precursor microRNA). After that, the precursor molecules are transported to the cytoplasm by Exportin-5 and Ran-GTP and undergo final processing step including the cleavage by Dicer and the formation of stem-loop duplex molecule structure which contains the single-stranded mature microRNA molecule and a microRNA* fragment. Finally, the duplex molecule structure is incorporated into the RNA-induced silencing complex (RISC), the microRNA* fragments are degraded, and mature microRNA molecules are formed. The mature microRNAs can display genic regulation role via recognizing and binding to the 3′-untranslated region of their target genes' mRNAs. *Note*: This figure is plotted according to ScienceSlides (version#2016).

targeting genes via recognizing and integrating into the 3′-untranslated region of these genes' mRNAs. On the basis of perfect or imperfect base-base complementarity of microRNAs-their targeting mRNA binding, one microRNA specifically regulates the expression of multiple mRNAs, and at the same time, one mRNA might be inhibited by multiple microRNAs. This indicates the specificity and diversity of microRNAs regulating gene expression. In the past decades, microRNAs are emerged as important players in a very wide range of physiological processes including cell differentiation, cell proliferation and apoptosis, cycle regulation, survival, detoxification, physiological timing, metabolism, angiogenesis, hormone secretion, and DNA damage repair (**Figure 1**). Furthermore, growing evidence has shown that microRNAs can also display a role in the etiology and pathogenesis of various cancers by targeting many oncogenes or tumor inhibitive genes (**Figure 1**) [24, 27, 29–32]. Recent several reports have exhibited that some microRNAs involve in the tumorigenesis and procession of hepatocarcinoma and may become new potential markers for hepatocarcinoma diagnosis and prognosis [24, 27, 29, 31, 32].

3. MicroRNAs as novel biomarkers for hepatocarcinoma diagnosis

3.1. Diagnostic potential of single microRNA for hepatocarcinoma

With increasing incidence and death rate of hepatocarcinoma, it is very expected to identify one or several diagnostic biomarkers (with both high sensitivity and specificity) such as microRNAs for this malignancy. Growing evidence has shown that the expression change of all microRNAs in the peripheral blood may have a unique advantage because they exhibit tissue specificity and relative stability and can also provide some specific cues for early and small hepatocarcinoma [8–14]. Until now, more than 30 circulating microRNAs have been identified to have diagnostic potential for hepatocarcinoma (**Table 1**). For example, microRNA-122 has been reported as a hepatic-specific microRNA, accounting for 70% of the total microRNAs in hepatic tissues. This microRNA, a high conservative microRNA between vertebrate species, is indicative of a regulator of fatty acid metabolism and playing a critical role in liver homeostasis and tumorigenesis [19, 33, 34]. Increasing evidence has shown that elevated serum amount of microRNA-122 is positively associated with the severity of hepatic diseases including hepatitis, fatty- and alcohol-related liver damage, and drug-induced hepatotoxicity [35–39]. Interestingly, this increasing serum expression of microRNA-122 is noticeable and indicated that it could serve as a potential biomarker for the detection of patients with hepatocarcinoma from healthy controls with about 85% of the area under the receiver operating characteristic curve (AUC), 80% of sensitivity, and 80% of specificity [40, 41]. These results indicate that the dysregulated miR-122 in the peripheral blood may be used as a potential marker for hepatocarcinoma diagnosis. Results from retrospective studies have suggested that the microRNA-200 family (consisting of microRNA-200a and microRNA -200b) is also a promising biochemical biomarker for hepatocarcinoma diagnosis because of its deregulation during the development of both hepatic fibrosis and hepatocarcinoma [42, 43]. The elevated plasma levels of microRNA-21 can distinguish patients with hepatocarcinoma from cases with chronic hepatitis (with 61.1% of sensitivity and 83.3% of specificity) or healthy controls (the corresponding sensitivity and specificity are 87.3 and 92.0%, respectively) [44]. This suggests that this biomarker may have higher diagnostic potential than AFP. Some

MicroRNAs	Source	Diagnostic relevance	Expression level	AUC (95% CI)	Sen (%)	Spe (%)	Refs
miR-12	Serum	HCCs (n = 101) vs. HCs (n = 89)	Upregulated	0.87 (0.81–0.93)	84.0	75.3	[41]
miR-122	Serum	HCCs (n = 101) vs. HCs (n = 89)	Upregulated	0.79 (0.71–0.86)	70.7	69.1	[41]
miR-223	Serum	HCCs (n = 101) vs. CHCs (n = 89)	Upregulated	0.86 (0.80–0.92)	80.0	76.5	[41]
miR-12	Serum	HCCs (n = 101) vs. HCs (n = 48)	Upregulated	0.91 (0.84–0.97)	80.0	95.6	[41]
miR-122	Serum	HCCs (n = 101) vs. HCs (n = 48)	Upregulated	0.93 (0.88–0.98)	80.0	91.2	[41]
miR-223	Serum	HCCs (n = 101) vs. HCs (n = 48)	Upregulated	0.88 (0.81–0.94)	80.0	75.0	[41]
miR-122	Serum	HCCs (n = 70) vs. HCs (n = 34)	Upregulated	0.87 (0.79–0.95)	81.6	83.3	[40]
miR-122	Serum	HCCs (n = 70) vs. CHCs (n = 45)	Upregulated	0.63 (0.52–0.74)	77.6	57.8	[40]
miR-21	Plasma	HCCs (n = 126) vs. HCs (n = 50)	Upregulated	0.77	61.1	83.3	[44]
miR-21	Plasma	HCCs (n = 126) vs. CHCs (n = 30)	Upregulated	0.95	87.3	92.0	[44]
miR-143	Serum	HCCs (n = 95) vs. CTLs (n = 245)	Upregulated	0.80 (0.68–0.92)	73.0	83.0	[46]
miR-215	Serum	HCCs (n = 95) vs. CTLs (n = 245)	Upregulated	0.82 (0.72–0.97)	80.0	91.0	[46]
miR-10b	Serum	HCCs (n = 27) vs. HCs (n = 50)	Upregulated	0.85 (0.76–0.94)	/	/	[51]
miR-10b	Serum	HCCs (n = 27) vs. CLDs (n = 31)	Upregulated	0.73 (0.60–0.86)	/	/	[51]
miR-106b	Serum	HCCs (n = 27) vs. HCs (n = 50)	Upregulated	0.82 (0.72–0.91)	/	/	[51]
miR-106b	Serum	HCCs (n = 27) vs. CLDs (n = 31)	Upregulated	0.71 (0.57–0.84)	/	/	[51]
miR-181a	Serum	HCCs (n = 27) vs. HCs (n = 50)	Upregulated	0.89 (0.81–0.97)	/	/	[51]
miR-181a	Serum	HCCs (n = 27) vs. CLDs (n = 31)	Upregulated	0.81 (0.70–0.92)	/	/	[51]
miR-206	Serum	HCCs (n = 261) vs. HCs (n = 173)	Upregulated	0.62 (0.55–0.68)	48.1	78.8	[52]
miR-143-3p	Serum	HCCs (n = 261) vs. HCs (n = 173)	Upregulated	0.76 (0.70–0.80)	68.1	83.3	[52]
miR-433-3p	Serum	HCCs (n = 261) vs. HCs (n = 173)	Upregulated	0.74 (0.67–0.80)	79.3	64.4	[52]
miR-1228-5p	Serum	HCCs (n = 261) vs. HCs (n = 173)	Upregulated	0.55 (0.44–0.60)	79.3	27.8	[52]
miR-199a-5p	Serum	HCCs (n = 261) vs. HCs (n = 173)	Downregulated	0.64 (0.57–0.71)	59.3	66.7	[52]

MicroRNAs	Source	Diagnostic relevance	Expression level	AUC (95% CI)	Sen (%)	Spe (%)	Refs
miR-122-5p	Serum	HCCs (n = 261) vs. HCs (n = 173)	Downregulated	0.70 (0.63–0.77)	48.9	82.2	[52]
miR-192-5p	Serum	HCCs (n = 261) vs. HCs (n = 173)	Downregulated	0.70 (0.62–0.77)	71.9	75.6	[52]
miR-26a-5p	Serum	HCCs (n = 261) vs. HCs (n = 173)	Downregulated	0.76 (0.70–0.82)	68.9	74.4	[52]
miR-206	Serum	HCCs (n = 261) vs. CCs (n = 233)	Upregulated	0.69 (0.62–0.77)	77.8	68.9	[52]
miR-143-3p	Serum	HCCs (n = 261) vs. CCs (n = 233)	Upregulated	0.66 (0.60–0.73)	60.7	72.7	[52]
miR-433-3p	Serum	HCCs (n = 261) vs. CCs (n = 233)	Upregulated	0.64 (0.58–0.71)	56.4	67.4	[52]
miR-1228-5p	Serum	HCCs (n = 261) vs. CCs (n = 233)	Upregulated	0.54 (0.47–0.61)	66.7	47	[52]
miR-199a-5p	Serum	HCCs (n = 261) vs. CCs (n = 233)	Downregulated	0.59 (0.52–0.66)	59.3	57.6	[52]
miR-122-5p	Serum	HCCs (n = 261) vs. CCs (n = 233)	Downregulated	0.75 (0.69–0.81)	48.9	90.2	[52]
miR-192-5p	Serum	HCCs (n = 261) vs. CCs (n = 233)	Downregulated	0.69 (0.62–0.75)	54.8	83.3	[52]
miR-26a-5p	Serum	HCCs (n = 261) vs. CCs (n = 233)	Downregulated	0.74 (0.68–0.81)	60.7	90.9	[52]
miR-16	Serum	HCCs (n = 105) vs. CTLs (n = 188)	Downregulated	/	72.1	88.8	[14]
miR-199	Serum	HCCs (n = 105) vs. CTLs (n = 188)	Downregulated	/	62.9	93.5	[14]
miR-199a	Serum	HCCs (n = 105) vs. CTLs (n = 188)	Downregulated	/	78.1	64.5	[14]
miR-375	Serum	HCCs (n = 78) vs. HCs (n = 156)	Downregulated	0.64 (0.56–0.74)	/	/	[53]
miR-199a-3p	Serum	HCCs (n = 78) vs. HCs (n = 156)	Downregulated	0.88 (0.83–0.94	/	/	[53]
miR-30c-5p	Plasma	HCCs (n = 8) vs. CTLs (n = 86)	Upregulated	/	/	/	[54]
miR-223-3p	Plasma	HCCs (n = 8) vs. CTLs (n = 86)	Downregulated	/	/	/	[54]
miR-202c-3p	Plasma	HCCs (n = 8) vs. CTLs (n = 86)	Upregulated	/	/	/	[54]
miR-17-57	Plasma	HCCs (n = 8) vs. CTLs (n = 86)	Upregulated	/	/	/	[54]
miR-4651	Serum	AHCCs (n = 279) vs. HCs (n = 338)	Upregulated	0.89 (0.86–0.92)	78.1	99.1	[55]
miR-4651	Serum	AHCCs (n = 279) vs. AHCs (n = 292)	Upregulated	0.82 (0.78–0.85)	78.1	85.3	[55]
miR-4651	Serum	AHCCs (n = 279) vs. ALCs (n = 32)	Upregulated	0.80 (0.71–0.88)	78.1	81.2	[55]

MicroRNAs	Source	Diagnostic relevance	Expression level	AUC (95% CI)	Sen (%)	Spe (%)	Refs
miR-4651	Serum	AHCCs (n = 279) vs. CTLs (n = 662)	Upregulated	0.85 (0.82–0.88)	78.1	92.1	[55]
miR-143	Serum	HCCs (n = 131) vs. HCs (n = 122)	Downregulated	0.83	80.3	82.4	[56]
miR-125b	Plasma	HCCs (n = 64) vs. HCs (n = 56)	Downregulated	0.89	90.0	80.0	[57]
miR-125b	Plasma	HCCs (n = 64) vs. CHBs (n = 63)	Downregulated	0.96	90.0	90.0	[57]
miR-125b	Plasma	HCCs (n = 64) vs. CCs (n = 59)	Downregulated	0.96	90.0	90.0	[57]
miR-150	Serum	HCCs (n = 120) vs. CHBs (n = 110)	Downregulated	0.88 (0.84–0.93)	79.1	76.5	[58]
miR-150	Serum	HCCs (n = 120) vs. HCs (n = 120)	Downregulated	0.93 (0.90–0.96)	82.5	83.7	[58]
miR-106b	Plasma	HCCs (n = 47) vs. CTLs (n = 61)	Upregulated	0.81	0.7	0.8	[59]
miR-200a	Serum	HCCs (n = 22) vs. HCs (n = 15)	Downregulated	0.82 (0.69–0.97)	/	/	[60]
miR-200a	Serum	HCCs (n = 22) vs. CCs (n = 22)	Downregulated	0.73 (0.56–0.89)	/	/	[60]
miR-143	Serum	HCCs (n = 95) vs. CHCs (n = 118)	Upregulated	0.62 (0.51–0.76)	78.0	64.0	[46]
miR-215	Serum	HCCs (n = 95) vs. CHCs (n = 118)	Upregulated	0.80 (0.67–0.95)	78.0	89.0	[46]
miR-143	Serum	HCCs (n = 95) vs. HCs (n = 127)	Upregulated	0.80 (0.68–0.92)	78.0	89.0	[46]
miR-215	Serum	HCCs (n = 95) vs. HCs (n = 127)	Upregulated	0.82 (0.72–0.97)	80.0	91.0	[46]
miR-101	Serum	HCCs (n = 67) vs. HCs (n = 30)	Downregulated	0.79 (0.69–0.87)	76.1	70.0	[61]
miR-483-5p	Serum	HCCs (n = 49) vs. HCs (n = 49)	Upregulated	0.91	75.5	89.8	[62]
miR-122a	Plasma	HCCs (n = 85) vs. HCs (n = 85)	Downregulated	0.71	70.6	67.1	[63]
miR-618	Urine	HCCs (n = 32) vs. CTLs (n = 74)	Upregulated	0.66	64.0	68.0	[47]
miR-650	Urine	HCCs (n = 32) vs. CTLs (n = 74)	Downregulated	0.65	72.0	58.0	[47]
miR-126	tumor tissue	HCCs vs. CAs	Upregulated	/	/	/	[48]

Abbreviation: miR, microRNA; HCCs, cases with hepatocarcinoma; CTLs, non-HCC controls (including healthy control and other nontumor controls); HCs, healthy controls; CCs, controls with liver cirrhosis; CHBs, patients with chronic hepatitis B; CLDs, cases with nontumor chronic liver diseases; Sen, sensitivity; Spe, specificity; AUC, the area under the receiver operating characteristic curve; CI, confidence interval; Refs, references.

Table 1. The microRNAs as diagnostic biomarkers for hepatocarcinoma.

other serum microRNAs, such as microRNA-15b, microRNA-130b, miR-143, and miR-215, are additional potential biomarkers that are significantly dysregulated in hepatocarcinoma [45, 46]. Noticeably, these biomarkers also exhibit their diagnostic potential for patients with early-stage hepatocarcinoma and/or negative-status AFP [45, 46].

Recently, some evidence has also exhibited that microRNAs in urine samples and liver tissues have screening potential for hepatocarcinoma (**Table 1**). Actually, the detection of five deregulated microRNAs, including microR-618, microRNA-625, microRNA-650, microRNA-532, and miR-516-5P, in the urine samples has already been used for screening patients with the early and small hepatocarcinoma from these with risk factors such as chronic virus hepatitis, liver cirrhosis, and dysplasia [47]. Barshack et al. [48] investigated differential diagnosis potential of microRNAs for discriminating hepatocarcinoma from metastatic tumors in the liver using custom microarray expression technique. In their study, they tested the distributed features of microRNAs among 144 tumor samples with or without metastatic adenocarcinoma and similar hepatocarcinoma in the morphology and immune types and found that microR-141 and microR-200c can promote non-hepatic epithelial phenotypes while microRNA-126 displays hepatic epithelial phenotypes. Higher expression of microRNA-126 is further shown in these tissue samples with hepatocarcinoma. Therefore, the change profiles of microRNAs in body fluids (such as urine) and tumor tissues may represent a kind of gold biomarkers for such cancers as liver carcinoma.

However, the specificity of a single microRNA identifying hepatocarcinoma is relatively poor. For example, the serum level of aforementioned liver-specific microRNA-122 is upregulated not only among cases with hepatocarcinoma but also among these with chronic virus hepatitis, liver cirrhosis, and fatty liver diseases caused by alcohol or non-alcohol [49, 50]. Evidence has shown that serum microRNA-122 does not discriminate patients with hepatocarcinoma from these with chronic hepatitis, although higher expression is observed among cancer cases [40, 41]. This indicates that more investigations on the basis of large size of samples and the prospective randomized controlled trials should help us for addressing these concerns.

3.2. Diagnostic potential of microRNA panel for hepatocarcinoma

Because hepatocarcinoma is a multifactor-induced highly complex malignant disease with heterogeneous feature, a combination of multiple microRNAs in place of a single microRNA may have higher accuracy for hepatocarcinoma discrimination. Several circulating microRNA panels have been reported to have higher early diagnostic value for hepatocarcinoma (**Table 2**) [47, 51, 52, 64–70]. For example, Lin et al. [70] preformed a three-stage study consisting of the discovery stage (including 6 cases with hepatocarcinoma and 8 cases with chronic hepatitis B), the training stage (including 108 cases with hepatocarcinoma, 51 cases with chronic hepatitis B, 47 cases with liver cirrhosis, and 51 healthy controls), and the validation stage (including 229 patients with hepatocarcinoma and 424 controls with or with nontumor liver diseases). In the first stage, they identified 31 different serum microRNAs between individuals with hepatocarcinoma and those with chronic hepatitis B using the TaqMan Array technique. Next, they validated these different microRNAs and constructed diagnostic panel containing miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505 on the basis of logistic regression model. Finally, the established serum microRNA panel was tested among

MicroRNA panel	Source	AUC (95% CI)	Sen (%)	Spe (%)	Diagnostic relevance	Refs
miR-10b + miR-106b + miR-181a	Serum	0.94 (0.89–0.99)	/	/	HCCs (n = 27) vs. HCs (n = 50)	[51]
miR-10b + miR-106b + miR-181a	Serum	0.91 (0.80–0.97)	/	/	HCCs (n = 27) vs. CLDs (n = 31)	[51]
miR-206 + miR-143-3p + miR-433-3p + miR-1228-5p + miR-199a-5p + miR-122-5p + miR-192-5p + miR-26a-5p	Serum	0.89 (0.85–0.94)	82.8	83.3	HCCs (n = 261) vs. HCs (n = 173)	[52]
miR-206 + miR-143-3p + miR-433-3p + miR-1228-5p + miR-199a-5p + miR-122-5p + miR-192-5p + miR-26a-5p	Serum	0.89 (0.84–0.94)	81.6	84.6	HCCs (n = 261) vs. CCs (n = 233)	[52]
miR-122 + miR-192 + miR-21 + miR-223 + miR-26a + miR-27a + miR-801	Plasma	0.86 (0.83–0.90)	68.6	90.1	HCCs (n = 204) vs. CTLs (n = 303)	[64]
miR-122 + miR-192 + miR-21 + miR-223 + miR-26a + miR-27a + miR-801	Plasma	0.89 (0.85–0.92)	81.8	83.5	HCCs (n = 196) vs. CTLs (n = 194)	[64]
miR-27b-3p + miR-192-5p	Serum	0.84 (0.78–0.89)	0.7	0.9	HCCs (n = 91) vs. CTLs (n = 91)	[65]
miR-92-3p + miR-107 + miR-3126-5p	Serum	0.97 (0.95–0.99)	/	/	HCCs (n = 115) vs. HCs (n = 40)	[66]
88-miRNA	Serum	1.00 (0.97–1.00)	100.0	99.2	HCCs (n = 261) vs. CCs (n = 233)	[67]
miR214-5p + miR-125b + miR-1269 + miR-375	Serum	0.95	96.9	83.2	HCCs (n = 224) vs. HCs (n = 84)	[68]
miR-122 + miR-885-5p + miR-29b	Serum	1.00	/	/	HCCs (n = 192) vs. HCs (n = 96)	[69]
miR-29a + miR-29c + miR-133a + miR-143 + miR-145 + miR-192 + miR-505	Serum	0.82 (0.77–0.87)	74.5	89.9	HCCs (n = 153) vs. CTLs (n = 199)	[70]
miR-29a + miR-29c + miR-133a + miR-143 + miR-145 + miR-192 + miR-505	Serum	0.88 (0.82–0.95)	85.7	91.1	HCCs (n = 49) vs. CTLs (n = 90)	[70]
miR-618 + miR-650	Urine	0.69	58.0	75.0	HCCs (n = 32) vs. CTLs (n = 74)	[47]

Abbreviation: miR, microRNA; HCCs, cases with hepatocarcinoma; CTLs, non-HCC controls (including healthy control and other nontumor controls); HCs, healthy controls; CCs, controls with liver cirrhosis; CHBs, patients with chronic hepatitis B; CLDs, cases with nontumor chronic liver diseases; Sen, sensitivity; Spe, specificity; AUC, the area under the receiver operating characteristic curve; CI, confidence interval; Refs, references.

Table 2. The microRNA panel as diagnostic biomarkers for hepatocarcinoma.

individuals from the training and validation cohorts. These data identified seven microRNAs and constructed a serum microRNA panel with an increasing diagnostic accuracy for hepatocarcinoma [AUC = 0.826 (0.771–0.880) for training set and 0.817 (0.769–0.865) and 0.884 (0.818–0.951) for two different validation sets, respectively]. Interestingly, a nest case-control study has further proved that this panel could be used to detect preclinical hepatocarcinoma as well as small-size, early-stage, and α-fetoprotein-negative disease.

Similarly, Jiang et al. [59] and Zhou et al. [64] also attempted to identify possible combination of different microRNAs for increasing diagnostic accuracy of hepatocarcinoma on the basis of different controls with or without liver diseases. They found that the panel consisting of miR-10b, miR-106b, and miR-181a as well as the combination of miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801 can improve detection of hepatocarcinoma. These reports indicate that the panel of microRNAs may have better performance than a single-microRNA assay.

3.3. Diagnostic potential of microRNAs binding with AFP for hepatocarcinoma

AFP has been regarded as the most important marker for hepatocarcinoma screening and diagnosis, ever since it was identified in the peripheral blood samples from patients with hepatocarcinoma in 1964 [8, 71, 72]. However, this marker is relatively unsatisfactory because of its low sensitivity and specificity. This is mainly because only 60–80% of cases with hepatocarcinoma show positive AFP, whereas about 40% of cirrhotic patients also exhibit different degree increasing level of serum AFP [73, 74]. Thus, AFP may not be a reliable hepatocarcinoma marker, especially for early-stage and/or AFP-negative hepatocarcinoma. On the basis of low sensitivity and specificity of AFP for hepatocarcinoma diagnosis, the American Association for the Study of Liver Disease Practice Guidelines has thrown it away for prognostic surveillance and tumor diagnosis [75]. However, recent studies have displayed that the combination of AFP in the peripheral blood and microRNAs in body fluids may improve the sensitivity and specificity of hepatocarcinoma diagnosis and increase their diagnostic potential [14, 47, 55, 58, 65–67, 69, 70, 76].

For example, Wu et al. [55] investigated the joint diagnostic value of serum microRNA-4651 and AFP for hepatocarcinoma in 279 hepatocarcinoma patients, 324 controls with liver injury, and 338 healthy controls. Their results imply that serum microRNA-4651 has higher expression level among cases with hepatocarcinoma (AUC of 0.85; sensitivity of 78.1% and specificity of 92.1%); this increasing expression also displays higher diagnostic potential than AFP at cutoff of 20 ng/mL (AFP20) (AUC = 0.80, sensitivity = 61.3%, and specificity = 98.8%) and of 400 ng/mL (AFP400) (AUC = 0.72, sensitivity = 43.0%, and specificity = 100.0%). Noticeably, the combination of serum microRNA-4651 with AFP significantly improves the discrimination power between patients with hepatocarcinoma and with chronic nontumor liver injury (AUC = 0.90, sensitivity = 83.2%, and specificity = 97.1%). Similar findings have also been observed in the analyses of combination of serum AFP and other microRNAs, such as miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, miR-505, miR-16, miR-195, and miR-199a [14, 47, 58, 65–67, 69, 70, 76]. Altogether, these data suggest that the combination of microRNAs with AFP may improve diagnostic potential of hepatocarcinoma.

4. Prognostic potential of microRNA for hepatocarcinoma

In the past decades, growing evidence has exhibited that microRNAs can act as prognostic biomarkers for hepatocarcinoma [56, 77–101], and **Table 3** summarizes these significantly affecting hepatocarcinoma outcomes. Functionally, the microRNAs affect hepatocarcinoma prognosis

MicroRNAs	Source	Expression level	Prognostic significance	HR (95%CI)	Refs
miR-122	Serum	Upregulated	Increasing levels correlate with poor OS	OS, 0.08 (0.03–0.22)	[120]
miR-1	Serum	Upregulated	Increasing levels correlate with poor OS	OS, 0.45 (0.23–0.86)	[121]
miR-122	Serum	Upregulated	Correlated with clinical chemistry parameters of hepatic necroinflammation, liver function, and synthetic capacity	/	[121]
miR-221	Serum	Upregulated	(1) Correlated with tumor size, cirrhosis, and tumor stage; (2) increasing levels decreased survival rate	/	[122]
miR-4651	Serum	Upregulated	Increasing levels correlate with poor OS and RFS	OS, 2.67 (1.61–4.42) RFS, 3.62 (1.49–8.81)	[55]
miR-1268a	Tumor tissues	Upregulated	Increasing levels correlate with poor OS and RFS	OS, 2.44 (1.82–3.23) RFS, 2.86 (2.08–3.85)	[115]
miR-24	Tumor tissues	Upregulated	Increasing levels correlate with poor OS and RFS	OS, 3.58 (2.34–5.46) RFS, 4.75 (2.66–8.47)	[77]
miR-429	Tumor tissues	Upregulated	Increasing levels correlate with poor OS and RFS	OS, 4.64 (2.56–8.41) RFS, 6.94 (3.19–15.08)	[78]
miR-143	Serum	Downregulated	Decreasing levels correlate with poor OS and RFS	/	[56]
miR-9	Tumor tissues	Upregulated	Increasing levels correlate with poor OS and RFS	/	[123]
miR-92b	Tumor tissues, serum	Upregulated	Increasing levels promoting tumor metastasis	/	[102]
miR-150	Serum	Downregulated	Increasing levels correlate with poor OS	0.45 (0.23–0.85)	[58]
miR-21	Serum	Upregulated	Increasing levels correlate with poor OS	2.23 (1.33–3.74)	[76]
20-miRNA signature	Tumor tissues	10 downregulated and 20 upregulated miRNAs	6 were risk factors and 14 were protective factors	OS, 2.75 (1.58–4.79)	[124]
miR-221	Tumor tissues	Upregulated	Increasing expression promotes metastasis-free survival	/	[125]

MicroRNAs	Source	Expression level	Prognostic significance	HR (95%CI)	Refs
miR-96	Tumor tissues	Upregulated	Increasing expression correlates with poor RFS	/	[126]
miR-92a	Tumor tissues	Downregulated	Decreasing expression correlates with poor RFS	RFS, 1.60 (1.00–2.50)	[79]
miR-22	Tumor tissues	Downregulated	Decreasing expression correlates with poor RFS	/	[80]
miR-500	Serum	Upregulated	Decreasing expression correlates with tumor resected	/	[127]
miR-375	Tumor tissues	/	Decreasing expression correlates with poor RFS	RFS, 3.273	[81]
miR-148b	Tumor tissues	Downregulated	Decreasing expression correlates with poor OS	OS, 1.86 (1.23–2.98)	[82]
miR-101	Tumor tissues	Downregulated	Decreasing expression correlates with poor OS and RFS	RFS, 2.56 (1.32–5.69) OS, 3.27 (1.18–6.92)	[83]
miR-19a	Tumor tissues	Downregulated	Decreasing expression correlates with poor OS and RFS	/	[84]
miR-210	Tumor tissues	Upregulated	Increasing expression correlates with poor OS	/	[80]
miR-224	Tumor tissues	Upregulated	Increasing expression correlates with poor OS and RFS	/	[85]
miR-29	Tumor tissues	Downregulated	Decreasing expression correlates with poor and RFS	/	[86]
miR-139-5p	Tumor tissues	Downregulated	Decreasing expression correlates with poor OS	/	[87]
miR-1	Tumor tissues	Downregulated	Decreasing expression correlates with poor OS and RFS	OS, 2.79	[88]
miR-199b-5p	Tumor tissues	Downregulated	Decreasing expression correlates with poor OS	/	[89]
miR-130b	Tumor tissues	Upregulated	Increasing expression correlates with poor OS and RFS	RFS, 4.00 (1.58–7.90) OS, 2.52 (1.02–7.90)	[90]
miR-9	Tumor tissues	Upregulated	Increasing expression correlates with poor OS and RFS	/	[91]
miR-25	Tumor tissues	Upregulated	Increasing expression correlates with poor OS and RFS	RFS, 1.62 OS, 2.18	[92]
let-7	Tumor tissues	Upregulated	Increasing expression correlates with poor OS	/	[93]

MicroRNAs	Source	Expression level	Prognostic significance	HR (95%CI)	Refs
miR-30a	Tumor tissues	Downregulated	Decreasing expression correlates with poor RFS	RFS, 3.2 (1.5–6.8)	[94]
miR-99a	Tumor tissues	Downregulated	Decreasing expression correlates with poor RFS	RFS, 1.60 (1.00–2.50)	[79]
miR-106b	Tumor tissues	Upregulated	Increasing expression correlates with poor OS	OS, 2.00 (1.13–6.98)	[95]
miR-130a	Tumor tissues	Downregulated	Decreasing expression correlates with poor OS	OS, 2.22 (1.10–4.46)	[96]
miR-19b	Tumor tissues	/	Increasing expression correlates with good OS	OS, 0.45 (0.24–0.85)	[97]
miR-148a	Tumor tissues	Downregulated	Decreasing expression correlates with poor OS	/	[98]
miR-372	Tumor tissues	Upregulated	Increasing expression correlates with poor OS and RFS	RFS, 6.83 OS, 9.53	[99]
miR-630	Tumor tissues	Downregulated	Increasing expression correlates with good OS and RFS	OS, 0.71 (0.26–1.92) RFS, 0.66 (0.33–1.35)	[100]
miR-100	Tumor tissues	Downregulated	Decreasing expression correlates with poor OS	OS, 1.66 (1.32–2.82)	[101]

Abbreviation: miR, microRNA; OS, overall survival; RFS, tumor recurrence-free survival; HR, hazard ratio; CI, confidence interval; Refs, references.

Table 3. The microRNAs as prognostic biomarkers for hepatocarcinoma.

via the following pathways: (1) promoting cancerous growth and proliferation [77, 78, 80, 83, 89, 98, 99, 102–113], (2) inhibiting cancerous apoptosis [77, 78, 86, 99, 101, 107–109, 111, 112, 114], (3) increasing microvessel density in the tumor tissues [77, 115], (4) affecting cell cycles [24, 25, 27, 28, 116–119], (5) increasing the risk of tumor metastasis [77, 115], and (6) decreasing the sensitivity of cancer cells to anticancer drugs [115]. For example, Lu et al. [115] investigated the prognostic potential of microRNA-1268a for hepatocarcinoma in 411 patients with hepatocarcinoma. Their results imply that microRNA-1268a expression in the cancerous tissues is significantly related to tumor features including tumor volume, stage and grade, and microvessel density. Results from multivariable factors analyses based on Cox regression models show that microRNA-1268a expression is independent of other known prognostic factors for hepatocarcinoma. Furthermore, transarterial chemoembolization (TACE) treatment can improve the prognosis of hepatocarcinoma patients with low microRNA-1268a expression, but not for those with high microRNA-1268e expression. These data imply that the dysregulation of microRNA-1268a can modify the response of cancer cells to antidrugs. Their following studies prove that upregulated microRNA-1268a inhibited while its downregulation enhanced doxorubicin

(an anticancer drug)-induced the death of tumor cells. Similarly, Liu et al. [77] and Huang et al. [78] investigated the roles of microRNAs, such as microRNA-24 and microRNA-429, in the tumorigenesis of liver cancer on the basis of analyses of hepatocarcinoma samples and genic toxicity induced by aflatoxin B1 and found the dysregulation of these microRNAs increased microvessel density and mutation frequency of TP53 gene possibly resulting from the loss of DNA repair capacity. Taken together, these reports indicate that microRNAs in body fluids and cancerous tissues may be important candidate biomarkers for hepatocarcinoma prognosis.

5. Further direction

In the past decades, the advance in pathological mechanisms of microRNAs regulating tumorigenesis and procession of hepatocarcinoma holds great promise for identifying whether microRNAs in body fluids (such as blood and urine) act as novel early diagnostic and prognostic biomarkers for this malignancy. However, we are still far from a comprehensive view of this kind of potentials. Although some hepato-specific microRNAs have been identified, microRNAs in body fluids may be from hemocytes and vascular endothelial cells and others from tissues and organs with high blood flow as well as hepatocarcinoma. This kind of heterogeneous origin indicates that the dysregulation of tumor-specific microRNA signatures may be concealed by microRNAs from other origins. Furthermore, well-standardized protocols of testing microRNAs have not been constructed or confirmed on the basis of the prospective, randomized controlled trials. Disclosing the different diagnostic and prognostic potential of microRNAs will greatly benefit our constructing high accurate diagnostic and prognostic models for hepatocarcinoma and will shed important light on the early diagnosis, tumor monitoring, and prognosis prediction for individuals with risk factors.

6. Summary

To conclude, the advances in technologies, including microarray PCR technology, high-throughput sequencing, and mass spectrometry, make it possible to identify new markers for hepatocarcinoma diagnosis and prognosis. On the whole, the microRNAs are a class of attractive markers and may replace known traditional serum markers such as AFP on the basis of the following reasons. First, because many circulating microRNAs is highly stable and readily detected in patients with hepatocarcinoma, they may have higher diagnostic potential (with high AUCs, sensitivity, and specificity) for hepatocarcinoma than AFP. Second, some microRNAs appear in the urine and can be utilized for screening patients with high-risk factors of hepatocarcinoma. Third, some dysregulated microRNAs in the body fluids can change with the different stages of hepatocarcinoma, indicative of their potential in monitoring tumor recurrence. Finally, different expressions of microRNAs are useful for treatment strategies such as TACE selection. Taken together, the dysregulated microRNAs in body fluids (including urine and blood) may be a kind of promised biomarkers for liver carcinoma diagnosis and prognosis because they are early detected and easily monitored.

However, there are several issues to be noted. First, research on the diagnostic and prognostic potential of microRNAs is still in the early stages, and challenges are noticeable in the clinical utilization of significant microRNAs. Second, in spite of these biomarkers that are discussed well, their therapeutic potential still remains unclear. Finally, although the diagnostic and prognostic potential of microRNAs is well evaluated on the basis of retrospective case-control studies, results from the prospective, randomized controlled trials are absent. Finally, because of the polygenic feature for hepatocarcinoma development, it is essential for a panel of biomarkers to determine high-risk individuals. Thus, the advances in the fields of microRNAs including their origins, stability, detection strategies, variant characteristics, and biofunctions in hepatocarcinoma will progress microRNAs in body fluids to become possible tools for hepatocarcinoma diagnosis and prognosis in the future.

Conflicts of interest and source of funding

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Abbreviations

AFP	α-fetoprotein
AFB1	aflatoxin B1
AUC	the area under the receiver operating characteristic curve
CT	computed tomography
HBV	hepatitis virus B
HCC	hepatocellular carcinoma
HCV	hepatitis virus C
MRI	magnetic resonance imaging

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Section 3

Treatment Modalities for Hepatocellular Carcinoma

Chapter 6

Oncogenic Secretory Clusterin: A Promising Therapeutic Target for Hepatocellular Carcinoma

Min Yao, Wenjie Zheng, Li Wang, Miao Fang, Dengfu Yao and Zhizheng Dong

Additional information is available at the end of the chapter

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Abstract

Oncogenic secretory clusterin (sCLU) is a stress-induced molecular chaperone that confers proliferative and survival advantages to hepatocellular carcinoma (HCC), plays a crucial role in cell proliferation, multiple drug resistance, metastasis, and tumor progression. However, the targeted effects and molecular mechanisms of sCLU for malignant tumor are still unknown. This chapter aims to review some progression of oncogenic sCLU as a promising therapeutic target for HCC. An English-language literature search was conducted using bibliographic databases on some valuable articles in focused review questions to analyze the interventions and findings of included studies using a conceptual framework. The positive rate of hepatic sCLU expression in cancerous tissues was significantly higher more than that in their surrounding non-cancerous ones at gene transcription level or at protein level, with increasing according to tumor-node-metastasis (TNM) staging. Abnormal expression of oncogenic sCLU associated with poor differentiation degree and TNM stage of HCC also has been considered as a valuable diagnostic or independent prognostic biomarker for HCC. Furthermore, silencing sCLU at mRNA level by specific shRNA or inhibition by OGX-011 suppressed the colony formation and proliferation of tumor cells with apoptosis increasing, cell cycle arrested, alterations of cell migration and invasion behaviors, decreasing phosphorylation level of Akt and GSK-3β in vitro, and significantly suppressing the xenograft tumor growth with decreasing expression of β-catenin, p-GSK3β, and cyclinD1 in vivo. The oncogenic sCLU expression was closely associated with tumor progression, and it should be a novel potential molecular-targeted therapy for HCC.

Keywords: hepatocellular carcinoma, secretory clusterin, targeted therapy

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [1]. Growing understanding of the multiple pathogenic factors including hepatitis B or C virus (HBV or HCV) infection, toxic, lipid accumulation, aflatoxin B1 intake, and so on with complex molecular mechanisms underlying HCC reveal that hepatocarcinogenesis is a multistep process including lots of activated or suppressed oncogenes or anticancer genes [2–4]. Some techniques for HCC therapy have experienced great progress. However, the prognosis of HCC patients is still very poor due to the high rates of tumor recurrence and metastasis. Effective therapy of HCC is dependent on early specific diagnosis, therefore, to provide optimal treatment for patients, more precise and effective markers are urgently needed in all phases of management from early detection to staging, treatment monitoring, and prognosis [5–7]. Numerous studies have shown the clinical utility of novel blood-based markers, such as circulating tumor cells, key signal molecules, long non-coding RNA, and microRNA with great potential for HCC [8, 9].

Molecular chaperones are proteins that response to cellular stresses including genotoxic agents, nutrient starvation, and heat shock, with cellular stresses-induced protein misfolding, aggregation, and denaturation [10, 11]. To date, only few specific markers such as hepatoma-specific γ-glutamyl transferase [12, 13], oncofetal antigen glypican-3 (GPC-3) [14, 15], hepatoma-specific alpha-fetoprotein (HS-AFP or AFP-L3) [16], member 3a of Wingless-type MMTV integration site family (Wnt3a) [17, 18], and molecular chaperones like heat shock proteins (Hsp27 or Hsp90) [19] and clusterin have been developed as valuable biomarkers for primary hepatocellular carcinoma (PHC) diagnosis and surveillance. The clusterin (CLU) that was first detected in HCC tissues by Tobe et al., who found that SP40-40 gene in hepatoma cells was located in human chromosome 8, also designated as apolipoprotein J (APOJ), SP-40, sulfated glycoprotein 2 (SGP2), and testosterone-repressed prostate message 2 (TRPM2) [20]. Following the detection of their complete cDNA cloning, sequencing and comparison, secretory CLU (sCLU) is found to be the mature isoform of cytoplasm endoplasmic reticulum (ER)-Golgi CLU, which is over-expressed in a wide variety of tumors with oncogenicity [21, 22]. Recently, the mechanisms of abnormal sCLU expression and its targeted effects for HCC have been explored [23, 24]. This article summarizes some progression of sCLU as a promising target for HCC gene therapy.

2. Gene structure and functions of sCLU

Human CLU gene is a single-copy gene on chromosome 8p21-p12 including 9 exons and 8 introns, encoding an mRNA of 2877 bp and translating to a polypeptide with 449 amino acids (a.a.) [25]. The secretory glycoprotein (1st a.a.) is a signal sequence of hydrophobic leader, and targets the ER protein. CLU gene encodes two isoforms with distinct functions as a result of alternative splicing and post-translational modifications: cytoplasm sCLU (75–80 kDa) and nuclear CLU (nCLU, 55 kDa), which is mainly located in the nucleus. The sCLU molecule is a

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highly conserved heterodimeric disulfide-linked 449 amino acid polypeptide that represents the major product of CLU gene. The ER-Golgi sCLU is considered to influence immune regulation, transformation, tissue remodeling, lipid transport, membrane recycling, complements cascade, DNA repair, cell adhesion, and cell-cell interactions, indicated that sCLU is widely distributed in tissues and body fluids involved in various physiological processes [11, 26, 27].

Multiple reports have shown that the cytoplasm sCLU is cytoprotective and anti-apoptotic [28, 29], whereas nCLU protein is proapoptotic. Abnormal oncogenic sCLU expression was reported to correlate closely with HCC progression, such as inducing epithelial-mesenchymal transition (EMT) [30], formation of multiple drug resistance (MDR) [31], distal metastasis of tumor cells, and malignant transformation of hepatocytes, interaction with oncogenes or suppressor genes, and related signal pathways (**Figure 1**) [32, 33]. Because of ischemic or hypoxic microenvironment existence in cancerous tissues, the sCLU are often adaptively over-expressed and closely related with increased tumorigenicity, metastatic potential, and MDR to chemotherapy. As a stress-induced chaperone that inhibits protein misfolding and aggregation in a manner similar to small heat shock proteins (HSPs) [34, 35], its promoter

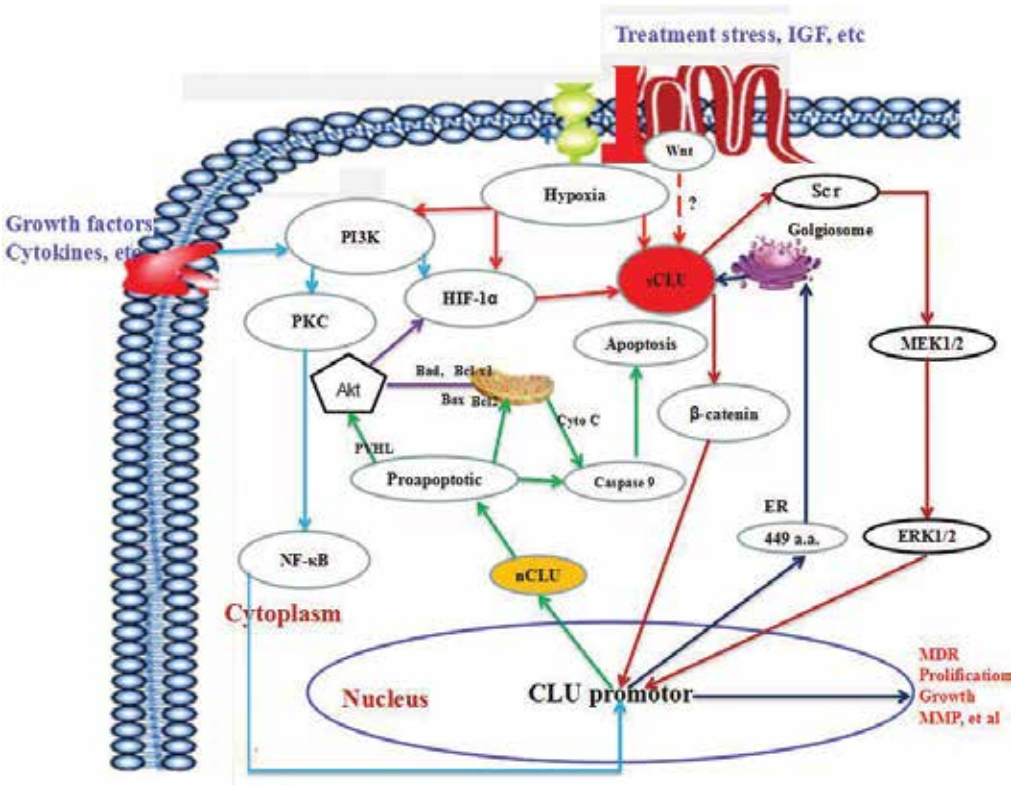

Figure 1. Potential mechanisms of hepatic sCLU in hepatocellular carcinoma. Cyto C, cytochrome c; ER, endoplasmic reticulum; Gene Reg., gene regulation; HIF-1α, hypoxia inducible factor-1α; IGF, insulin-like factor; MDR, multiple drug resistance; MMP, matrix metalloproteinase; nCLU, nuclear clusterin; NF-κB, nuclear factor-κB; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; Scr, sarcoma gene; sCLU, secretory clusterin.

region contains an element recognized by heat shock factor 1 (HSF-1) [36]. Cytoplasm sCLU-inhibited apoptosis by interacting with activated Bax, and protects HCC cells from ER stress-induced apoptosis through a physical interaction with glucose-regulated prptein78 (GRP78) [29, 37].

Hepatic sCLU has been confirmed that it was physically associated with eukaryotic translation initiation factor 3 subunit I (EIF3I), and might protect EIF3I protein from degradation. A positive correlation was founded between sCLU and EIF3I, and both of their functions might be as a cooperative unit in HCC. The levels of sCLU and EIF3I expression were investigated in HCC using tissue microarray (TMA) and the patients with high EIF3I level exhibited poor prognosis. After silenced EIF3I, Akt phosphorylation was significantly inhibited. The EIF3I-Akt complex could prevent PP2A-mediated dephosphorylation, which in turn led to a constitutive Akt signal activation, suggesting that the CLU-EIF3I complex might prevent EIF3I degradation, and then contribute to Akt upregulation [33, 38].

3. Biological behaviors of sCLU expression in cancerous tissues

Although great efforts have been made to explore molecular mechanism of HCC invasion and metastasis in the past decade [39–41], the mechanism of HCC remains incompletely understood. The alterations of sCLU expression at messenger RNA (mRNA) or protein level were investigated in HCC- and their non-tumorous tissues (NT) with self-control [42]. The overall level of sCLU mRNA in the HCC group was 75% up-regulated, 7.5% down-regulated, and 17.5% non-changed. Although no significant difference of the sCLU mRNA level at staging I was found between NT and HCC, they were drastically up-regulated expression from staging II to IV. The staining of sCLU mainly presented in the cytoplasm at protein level in HCC and their NT tissues were analyzed by tissue microarray (TMA) with immunohistochemistry (IHC). Its incidence in the HCC group (73.3%), with 37.5, 68 and 88.9% at staging I, II and III & IV, was significantly higher than that in the NT group (23.3%), respectively. The levels of sCLU protein consistent with their mRNA expression were gradually upregulation with increasing HCC staging [42, 43], indicated that high sCLU should be a valuable biomarker to distinguish malignant from benign liver nodular lesions [44].

The sCLU as a functional homolog of HSPs is a stress-induced chaperone that confers proliferative and closely associates with poor prognosis of HCC. Recurrence and metastasis are the most causes of poor prognosis of HCC. Clinicopathological features of sCLU revealed that its high expression was significantly linked to poor differentiation and advanced TNM stage [45]. There was a trend toward a poorer overall survival in HCC with high sCLU expression. Besides, survival time of HCC with high TNM stage was significantly shorter than that of cases with low stage. Moreover, in the subset of HCC patients with III and IV stage, high sCLU expression was prone to result in a shorter survival time (**Table 1**). There is a closely positive correlation between abnormal sCLU expression and HCC. High sCLU expression has more invasive phenotype for HCC [46]. The upregulation of sCLU is associated with HCC progression by contributing to angiogenesis, chemoresistance, cells survival, and metastasis.

Group		n	Pos. n (%)	χ^2 value	*P* value
AFP (μg /L)	≤50	37	31 (83.78)	1.733	0.118
	>50	38	27 (71.05)		
Portal vein invasion	With	7	6 (85.71)	0.309	0.578
	Without	68	52 (76.47)		
HBsAg	Negative	46	36 (78.26)	0.058	0.809
	Positive	29	22 (75.86)		
Tumor size	≤5 cm	45	33 (73.33)	1.027	0.311
	>5 cm	30	25 (83.33)		
Liver cirrhosis	With	57	45 (78.95)	0.353	0.552
	Without	18	13 (72.22)		
Lymph node metastasis	With	23	22 (95.65)	6.351	0.012*
	Without	52	36 (69.23)		
Differentiation	Well & moderate	58	43 (74.14)	1.491	0.222
	Poor	17	15 (88.24)		
Gross classification	Unifocal	62	46 (74.19)	1.744	0.187
	Multifocal	13	12 (92.31)		
TNM	I & II	45	30 (66.67)	7.683	0.021*
	III & IV	30	28 (93.33)		
Child degree	A	44	30 (68.18)	5.086	0.024*
	B&C	31	29 (90.32)		
5 years' survival	No	51	43 (84.31)	4.430	0.035*
	Yes	24	15 (62.50)		

Pos. n (%), positive number (%). *P<0.05

Table 1. Clinicopathological features of hepatic sCLU expression in HCC.

Growing evidences showed that sCLU with molecular chaperones played an important role in MDR formation, cells proliferation, metastasis of HCC [47]. Furthermore, univariate and multivariate analyses indicated that sCLU might be an independent prognostic indicator, in line with the factor of lymph node metastasis.

4. Circulating sCLU as diagnostic marker

The observations were in accordance with the early literature showing that upregulation of sCLU-positive expression might be associated with poor clinical outcome in HCC

patients [21, 48]. According to previous clinical studies, average serum sCLU level was significantly higher in the HCC group more than that in any of groups with cirrhosis, chronic hepatitis, or healthy control (**Table 2**). The area under receiver operating characteristic (ROC) curve and diagnostic sensitivity were 0.75 and 74.7% in serum sCLU, and 0.74 and 58.7% in serum AFP, respectively. The incidence of both combining detection rose up to 90.7% for HCC diagnosis (**Table 3**). High-circulating sCLU levels were observed in HCC patients, consistent with a recent study using a three-step serum proteome analysis, which showed that serum sCLU levels in HCC were significantly higher than those in benign liver diseases [42].

CLU is related to reverse cholesterol transport, platelet degranulation and human immune response pathways [49]. Protein-protein interaction analysis and pathway assessment showed a closed molecular relationship between cirrhosis and HCC [50]. Serum samples collected from HCCLM3-R metastatic HCC tumor model at specific stages of metastasis (1., 3 and 6 weeks) were subjected to iTRAQ labeling followed by 2DLC-ESI-MS/MS analysis. Circulating sCLU was significantly up-regulated during cancer progression and metastasis. The expression of sCLU was significantly higher in metastatic HCC cells and samples from HCC patients. Serum sCLU was highly increased with tumor size, tumor number, lymph node infiltration (**Table 4**) [42], and showed that the ROC area under curve value was 0.95 in sCLU more than that (0.85) in AFP. If 128 μg/mL as a cutoff value, the sensitivity or specificity of serum sCLU level for predicting HCC was 90 or 87%, respectively. The data indicated that circulating sCLU is a promising molecular marker of diagnosis or predicting metastasis for HCC [22, 51, 52].

Group	n	Mean ± SD	Positive n (%)	χ^2 value	*P* value
sCLU		**μg/mL**			
HCC	75	119.21 ± 16.67	56 (74.67)		
LC	30	97.78 ± 19.06	8 (26.67)**	20.744	<0.001
CH	30	106.30 ± 19.22	12 (40.00)**	11.285	<0.001
NC	36	89.96 ± 7.27	0 (0.00)**	54.249	<0.001
AFP		**ng/mL**			
HCC	75	2177.32 ± 3757.99	44 (58.67)		
LC	30	126.84 ± 244.76	10 (33.33)*	5.505	0.019
CH	30	30.27 ± 50.09	5 (16.67)**	15.188	<0.001
NC	36	7.1 ± 3.50	1 (2.78)**	31.520	<0.001

Serum sCLU values >104.0 μg/mL or AFP values >50 ng/mL were considered positive.

sCLU, secretory clusterin; LC, liver cirrhosis; CH, chronic hepatitis; NC, normal control; and AFP, α-fetoprotein.

*$P < 0.05$, compared with HCC group.

**$P < 0.01$, compared with HCC group.

Table 2. Serum sCLU or AFP levels among patients with chronic liver diseases and comparative analysis of both diagnostic values for HCC.

	sCLU level > 104.2 μg/mL (%)	AFP level > 50 ng/mL (%)	Both (%)
Sensitivity (%)	74.67	58.67	90.67
Specificity (%)	66.67	75.00	60.00
Accuracy (%)	71.11	65.93	77.03
Positive predictive value (%)	73.68	74.58	73.91
Negative predictive value (%)	67.80	59.21	83.72

AFP, α-fetoprotein; sCLU, secretory clusterin.

Table 3. Assessment diagnostic validity of serum sCLU or AFP level for HCC.

Group	n	Average (μg/mL)	*t* value	*P* value	Positive, n (%)	χ^2 value	*P* value
Age (years)							
≤60	47	118.09 ± 16.45	0.751	0.455	33 (70.21)	1.320	0.251
>60	28	121.09 ± 17.16			23 (82.14)		
AFP (μg/L)							
≤50	31	120.15 ± 17.12	0.405	0.686	24 (77.42)	0.212	0.645
>50	44	118.55 ± 16.50			32 (72.73)		
Portal vein invasion							
With	28	119.34 ± 14.52	0.050	0.960	22 (78.57)	0.360	0.548
Without	47	119.14 ± 17.97			34 (72.34)		
Tumor size							
≤5 cm	34	114.63 ± 18.10	2.221	0.029[a]	21 (61.76)	5.473	0.019
>5 cm	41	123.00 ± 14.52			35 (85.37)		
Lymph node metastasis							
Without	44	114.84 ± 15.21	2.826	0.006[b]	29 (65.91)	4.316	0.038
With	31	125.40 ± 16.91			27 (87.10)		
Differentiation							
Well & moderate	57	117.43 ± 16.34	1.662	0.101	42 (73.68)	0.121	0.728
Poor	18	124.84 ± 16.90			14 (77.78)		
Gross classification							
Unifocal	36	114.02 ± 18.57	2.698	0.009[c]	23 (63.89)	4.251	0.039
Multifocal	39	124.00 ± 13.20			33 (84.62)		

Group	n	Average (μg/mL)	*t* value	*P* value	Positive, n (%)	χ^2 value	*P* value
TNM stage							
I & II	31	113.93 ± 14.36	2.375 0.02[d]		20 (64.52)	2.878	0.09
III & IV	44	122.93 ± 17.31			36 (81.82)		
Child classification							
A	55	117.60 ± 14.56	1.395	0.167	40 (72.73)	0.410	0.522
B&C	20	123.63 ± 21.23			16 (80.00)		

AFP, α-fetoprotein; TNM, tumor node metastasis.
[a]With the tumor size ≥5 cm group.
[b]The lymph node metastasis group.
[c]With the multifocal group.
[d]With TNM III & IV group.

Table 4. Clinicopathologic features of serum sCLU expression in HCC patients (Mean ± SD).

5. Application prospect of targeting CLU gene

5.1. Antisense oligonucleotide (ASO) therapy

ASO is a useful technique to inhibit specific-targeted CLU genes, with a small synthetic natural nucleic acid analogue, that can complementary to CLU mRNA that induce degradation or inhibit translation into protein [52]. It is considered to be a potent inhibitor of sCLU expression in vitro, in vivo, and in human clinical trials, with no apparent effect on the expression of nCLU. Custirsen (OGX-011, 5′-CAGCAGCA GAGTCTTCATCAT-3′, 50 nM) is a novel 2′-methoxy-ethyl-modified phosphorothioate ASO, which is a 21-nucleoside complement to target the translation initiation site of CLU gene exon II mRNA translation initiation site with one CpG motif [53]. Hence, OGX-011 plays the role of chemosensitization by influencing the anti-apoptotic protein sCLU instead of the proapoptotic protein nCLU. Xiu et al. have reviewed the current state of research on clusterin, to predict future research directions and to analyze the potential of the clinical application of custirsen in HCC [54]. However, the median overall survival of HCC cases were ms 23.4 mo in the group of the OGX-011 combining anti-cancer drugs (docetaxel/prednisone) and ms 22.2 mo in the other group of cases with docetaxel/prednisone alone. No significant difference was found between two groups. Some potential key factors might contribute to its results, and still want to do more clinical trials to be ongoing [55].

5.2. Reversal MDR by specific shRNA-targeted sCLU

Resistance of tumor cells to chemotherapy continues to be a major clinical obstacle to extend the survival rate of patients with HCC. Recently, one of the major strategies for liver cancer is surgical resection with adjuvant anti-HCC drug chemotherapy [10]. However, the HCC patients always tend to acquire MDR during tumor progression. MDR-related P-glycoprotein (P-gp), encoded by MDR1 gene is positively linked closely with chronic liver diseases, because of its

drug efflux via ATP-binding cassette (ABC) family transporters, which can decrease intracellular concentration of anti-HCC drugs. Targeting sCLU to sensitize cancer cells to chemotherapy has become an attractive new strategy for cancer treatment. Previous studies found sCLU expressed in line with P-gp via immunohistochemical staining. The sCLU expression was analyzed in human hepatoma cells and chemoresistant counter-part HepG2/ADM cells. After transfection of shRNA-1 (5′-GTAAGTACGTC AATAAGGA-3′) into HepG2/ADM cells, the inhibition of CLU expression was 73.68% at mRNA level with obvious enhancement in chemosensitivity, and increasing apoptosis induced by doxorubicin [56]. Knockdown CLU also significantly decreased the drug efflux pump activity through the depression of MDR1/P-gp. Moreover, silencing CLU led to downregulation of β-catenin, suggesting that downregulation of CLU might be a key point to reverse MDR of HepG2/ADM cells [57].

5.3. Blockade-related pathway

5.3.1. PI3K/Akt/NF-κB pathway

Previous data revealed that CLU promoted cell survival through the PI3K/Akt pathway and induced MMP-9 expression via ERK1/2 and PI3K/Akt/NF-κB pathways [32]. CLU could increase p-Akt and MMP13 expression. A positive correlation between CLU expression and p-Akt level was observed in cohort of HCC tissues. Where CLU knockdown using OGX-011 significantly decreased p-Akt and MMP13 levels and suppressed HCC metastasis in two metastatic models through inhibiting EIF3I/Akt/MMP13 signaling. The related signaling molecule blockade of the PI3K-Akt pathway could significantly inhibited MMP13 expression in human HepG2-CLU or HCCLM3 cells [38, 58]. Decreased level of CLU accompanied with downregulation of MMP13 and p-Akt was observed in tumors derived from HCCLM3-shCLU group, revealed that p-Akt level was significantly correlated with poor prognosis and indicated that CLU may play a crucial role in HCC metastasis [38].

5.3.2. Wnt/β-catenin pathway

Wnt canonical pathway is often constitutively active in neoplastic cells, although, normally β-catenin is negatively regulated by GSK-3β that phosphorylates β-catenin to drive it for proteosomal degradation [30, 59]. Previous data emphasized sCLU modification after being exposed to Wnt/β-catenin inhibitor, and expressions of the crucial β-catenin and GSK-3β genes were detected in cases of sCLU depletion. The data indicated that sCLU suppression might lead to the inhibition of Wnt/β-catenin pathway in reverse. Hence, sCLU might play an important role in chemoresistance of HepG2/ADM cells together with Wnt/β-catenin signaling molecules [31, 56].

5.3.3. IGF-1/IGF-1R/Src/Mek/Erk pathway

Hepatic sCLU is a general genotoxic stress-induced, prosurvival gene product implicated in cancer [36, 37]. The regulatory signal transduction processes that control sCLU expression, the induction of sCLU is delayed, peaking 72 h after low doses of ionizing radiation, and is dependent on up-regulating IGF-1 and phosphorylation-dependent IGF-1R activation [23, 27] that stimulates the downstream Src-Mek-Erk signal transduction cascade to ultimately

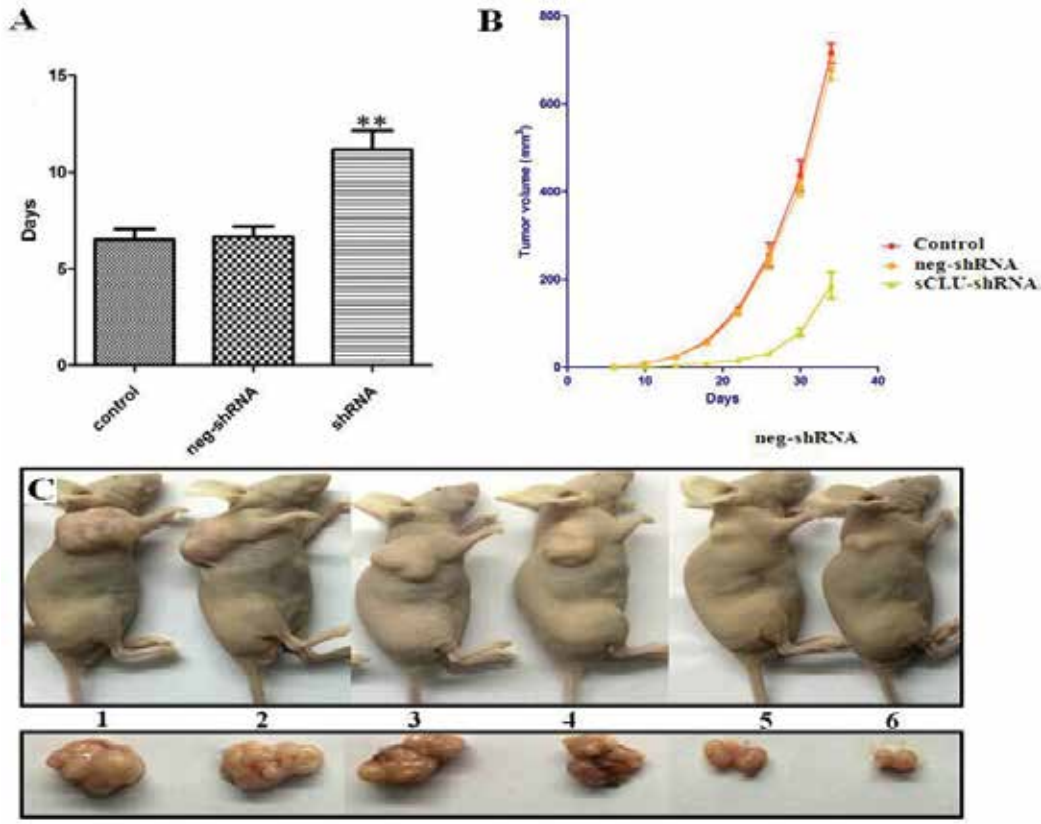

Figure 2. Inhibition of sCLU gene transcription by specific shRNA-1 on effects of forming time, growth curve and size of orthotopic xenograft tumors. The control, neg-shRNA, or sCLU-shRNA groups were injected with the plasmid contained specific shRNA-1 into nude mice. (A) The forming time of the orthotopic xenograft tumors from each group. (B) The growth curves of the xenograft tumors from each group. And the tumor volumes were measured at the indicated time point. (C) The representative photographs of the nude mice and corresponding dissected tumors from each group. 1 and 2, the xenografts in the control group; 3 and 4, the xenografts in the neg-shRNA group; 5 and 6, the xenografts in the sCLU-shRNA group. The data are presented as the mean ± SD. Compared with the control group, $^{**}P < 0.01$.

transactivate the early growth response-1 (Egr-1) transcription factor. Thus, the ionizing radiation exposure causes stress-induced IGF-1R-Src-Mek-Erk-Egr-1 activation that regulates the sCLU prosurvival cascade pathway for radiation resistance in HCC therapy [60].

5.4. Suppressed HCC growth in vivo by silencing sCLU

The inhibition of sCLU gene transcription by specific shRNA-1 on effects of forming time, growth curve, and size of orthotopic xenograft tumors after sacrifice of the mice at the 34th day with injection are shown in **Figure 2**. The mean weight of the xenograft tumors in the shRNA-1 group was significantly less than that of the control or NC-shRNA group, respectively. The curves of xenograft tumor growth indicated that tumor sizes in the shRNA-1 group with lower mRNA level were significantly smaller less than those of the control or NC-shRNA group [31, 46]. Consistently, the sCLU protein expression in the shRNA-1 group was also

lower than that in the control or the NC-shRNA group; and the sCLU staining in the control or NC-shRNA group was stronger than that in the shRNA-1 group by immunohistochemistry [46]. Specific shRNA-mediated downregulation of sCLU resulted in a reduced migratory capacity in HCC cell lines, as well as a reduction in pulmonary metastasis *in vivo* [38]. Overexpression of sCLU in HepG2 cell line showed increased cell migratory ability. In addition, sCLU also plays an important role in the regulation of TGF-β1-smad3 signaling pathway, suggested that oncogenic sCLU might promote HCC metastasis via the induction of EMT process and could be a promising candidate target for HCC therapy [32, 61–63].

6. Conclusions

In conclusion, the upregulation of sCLU expression at early staging of HCC is considered to promote tumor development, which may be related to the phosphorylation of AKT/GSK-3β. An increasing number of reports have provided evidence that sCLU level could be a novel biomarker for HCC diagnosis and prognosis, and there will be of great significance for the individualized treatment in HCC patients. The sCLU regulating signaling pathways could be critical to unraveling the solution for MDR in HCC. Therefore, silencing sCLU gene transcription and inhibiting sCLU expression by specific Custirsen inhibition have provided a new mechanism insight into molecular-targeted therapy for HCC in injected- or orthotopic model, indicated that sCLU gene would be a potential molecular-targeted for HCC therapy. Further study found that sCLU contributed to HCC migration and EMT *in vitro*, and metastasis *in vivo*. Although additional preclinical and clinical trials are necessary to explore the sCLU role in HCC, targeting the oncogenic sCLU could validate the approach as a systemic therapy to increase chemotherapy sensitivity.

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Abbreviations

AFP alpha-fetoprotein

HCC hepatocellular carcinoma

IHC immunohistochemistry

MDR multiple drug resistance

miRNA	microRNA
sCLU	secretory clusterin
shRNA	short hairpin RNA
TMA	tissue microarray
VEGF	vascular endothelial growth factor
Wnt	Wnt/β-catenin signaling pathway

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Chapter 7

Minimally Invasive Therapies for Hepatocellular Carcinoma: Mechanisms of Local Control and Systemic Immunologic Response

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Additional information is available at the end of the chapter

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Abstract

Minimally invasive treatments for hepatocellular carcinoma (HCC) are a cornerstone in the management of this challenging disease. For many years, percutaneously guided ablative techniques, such as radiofrequency ablation (RFA), cryoablation, and microwave ablation (MWA), have successfully treated many different solid malignancies including HCC. Since the initial implementation of these ablative techniques, there have been many advances in the design, technique, and patient selection as well as investigation into the body's response to treatment. The mechanisms of thermal-based ablative techniques, advantages and disadvantages of each technique, subsequent immunologic response following ablation, and advances in care that utilize combination therapy to potentiate the immunologic response creating a robust and long-term immunity to HCC are outlined in this chapter.

Keywords: hepatocellular carcinoma (HCC), immunotherapy, immunologic, response, immune, cancer, carcinoma, oncology, radiofrequency, microwave, ablation, cryoablation

1. Introduction

Hepatocellular carcinoma (HCC) is the most rapidly increasing type of cancer in the United States due to viral hepatitis and various forms of liver cirrhosis. HCC is resistant to traditional chemotherapy and often is not amenable to surgical resection due to factors involving the primary tumor or patient comorbidities [1, 2]. Thus, minimally invasive therapies for the treatment of malignant liver tumors have become a cornerstone of treatment. These minimally invasive techniques, including radiofrequency ablation (RFA), microwave ablation (MWA),

and cryoablation (cryo), have been shown to have distinct advantages over traditional treatment methods. These methods are not only able to locally control the malignancy through cellular necrosis and apoptosis but also potentially trigger systemic immune responses [3–6]. Additionally, these minimally invasive techniques offer other advantages such as lower morbidity, preservation of healthy tissues, lower cost, and decreased hospitalization time relative to surgical resection [5]. In this chapter, the mechanisms, advantages, disadvantages, synergism, and immunologic responses to the techniques outlined above are discussed.

2. Radiofrequency ablation

2.1. Overview

Radiofrequency ablation (RFA) is a minimally invasive technique used to thermally ablate targeted lesions in a variety of tissues. RFA is performed by percutaneously inserting one or more probes using various forms of image guidance, such as computed tomography (CT) or ultrasound (US). RFA may also be performed through other approaches such as laparoscopy and open surgery. The number of probes used is based on multiple factors such as the size of the lesion, the impedance of the targeted region, and surrounding structures such as blood vessels and lymphatic channels. In addition to the placement of RFA probes, one or more grounding pads are also placed on the patient. These grounding pads are located at a distant site from the probes. For example, a common practice is to place multiple grounding pads on both thighs when using RFA to ablate lesions located in the liver. Once the placement of the RFA probes has been confirmed with image guidance, an alternating current is generated by a power source between the probes and the grounding pads. This alternating current creates the thermal ablative region by causing ions to oscillate, generating frictional heat. RFA can reliably generate temperatures of 60–100°C in the targeted region leading to focal hyperthermic injury to the nearby cells. When temperatures reach 60°C or higher, instant cell death occurs, and at temperatures above 100°C, charring of surrounding tissues occurs. These two temperature points are crucial to the procedure because the operator can be certain that cell death has occurred in the regions >60°C, but it is also important to monitor the temperatures so that they do not increase too quickly or reach >100°C. If the temperature becomes too high, charring of the tissues occurs, which increases the impedance significantly and causes the technique to lose efficacy by diminishing the ablative zone substantially [7]. Additionally, at temperatures above 110°C, vaporization of the tissues occurs. Vaporization also increases the impedance of the tissues limiting the ablative zone [8].

The ablated area with RFA can be divided into three zones: central, transitional, and the unaffected surrounding parenchyma [8]. The central zone is the area directly surrounding the RFA probe. In this zone, the temperatures are the highest, typically >50–60°C, leading to coagulative necrosis of the cells in this region. The cells in this zone immediately undergo irreversible injury through protein denaturation of both the cytosolic, nucleic, and mitochondrial enzymes leading to coagulative necrosis. In addition to protein denaturation, the cell membrane integrity is also compromised. The higher temperatures in the central zone lead

to changes in fluid permeability through destruction of the membrane actin filaments. These membrane changes result in an intracellular fluid shift and subsequent cytolysis [5, 8].

Cells in the transitional zone are heated through conductive heat transfer from tissues in the central zone. This conductive heat transfer produces a sharp temperature gradient with average lower temperatures ranging from 40 to 45°C [5]. Cells within the transitional zone experience thermal injury, but since temperatures of 50°C are not reached, these cells do not undergo immediate cellular death [9]. Rather, the cells' metabolic processes and DNA repair mechanisms are impaired, which trigger specific changes that eventually lead to apoptosis or eventual cellular recovery. Other proposed mechanisms of cellular death include ischemia from vascular damage, reperfusion injury, and cytokine release and subsequent immunologic response to the damaged cells. Due to these changes, a complete response to ablation in this region will take several days to fully develop. This region also undergoes reactive hyperemia in response to the damage. The combination of hyperemia and increased cellular susceptibility creates a favorable environment to use liposomal chemotherapeutics. Liposomal chemotherapeutics will accumulate in the region due to the hyperemia and have increased activity on the already susceptible tumor cells. Since very few of the cells in this region are completely denatured, the transitional zone plays a critical role in the immunologic response, which will be discussed in more detail [5, 6, 8].

Surrounding parenchyma is not left totally unaffected by RFA. While the cells within this zone will not undergo cellular changes, necrosis, or apoptosis, there are several processes that will occur. There is an upregulation of various factors, presentation of antigens to antigen-presenting cells (APCs), and stimulation of the immune system, which will be discussed more in depth in later sections. Additionally, hyperemia occurs which can result in reperfusion injury [5, 6].

All of the above processes are dependent on a multitude of different factors such as the tumor composition, the surrounding parenchyma, the rate at which the energy is applied, and surrounding anatomic structures. The majority of the data on the effects of hyperthermia have been generated from literature on low-temperature hyperthermia that was applied uniformly over longer periods.

2.2. Patient selection

Traditionally, hepatic resection (HR) has been regarded as the first-line treatment for HCC, and RFA was typically reserved for patients with non-resectable disease. However, RFA has become a first-line treatment for early-stage HCC in patients with non-resectable disease, metastatic disease, recurrent HCC after HR, and for patients who are unable or are unwilling to undergo surgery [10]. RFA is best used in patients who have a solitary nodule <5 cm measured in the greatest dimension or less than three nodules all measuring <3 cm in the greatest dimension. RFA is most effective when treating HCC lesions that are ≤2 cm measured from the largest dimension. The reason it is more effective in these smaller lesions is that ablation margins of >4–5 mm can be easily obtained [1, 10]. Histologic and prospective studies have shown that the sensitivity of CT for detecting remnant neoplasm is anywhere between 36 and

44% [11, 12]. Thus, the clinician cannot readily rely on imaging to confirm that the lesion has been fully treated during or after the procedure making pre-procedure planning and patient selection crucial. Meta-analysis and systematic reviews have also shown that the efficacy of RFA and HR when used to treat lesions <5 cm is similar. There is no difference in 1-year overall survival; however, there is a difference in the 3- and 5-year survival. HR offers greater 3- and 5-year survival when compared to RFA as well as 1-, 3-, and 5-year disease-free rates. It has been postulated that these findings are due to how each treatment method works. With RFA, the primary lesion is directly targeted with minimal damage to the surrounding tissues, which may leave satellite lesions that would have been removed with HR. Additionally other factors come into play with RFA such as the shape and distribution of the ablation zone. However, RFA has been shown to have fewer complications during and after the procedure, shorter hospital stays, and is considered safer and less invasive than HR. While HR has a significant role in the treatment of HCC, there are limited studies comparing RFA to HR [10, 13, 14]. At the current time, it cannot be confirmed which treatment is superior to the other in treating early-stage HCC. It is up to the treating clinician to determine which treatment is best. RFA should be considered as a first-line treatment in specific patients with small solitary lesions <5 cm; patients with less than three lesions that are <3 cm; patients with non-resectable, metastatic, or recurrent disease; patients who elect for non-operative or a minimally invasive approach; and patients who are non-operative due to medical comorbidities [10].

2.3. Advantages and disadvantages of RFA

Of all the thermal ablative techniques for treating HCC, RFA has been the most researched. There are many technical advantages using RFA for the treatment of HCC. The most obvious advantage of RFA as well as other thermal ablative techniques is the ability to treat a wide variety of patients while sparing normal liver parenchyma.

However, an important consideration with RFA and other thermal ablative techniques is the "heat sink effect" of surrounding anatomic structures. The heat sink effect is caused when the desired ablative region contains or is abutted by larger vessels that result in heat dissipation. This dissipation can ultimately lead to temperatures not reaching cytotoxic levels in the lesion [15–17]. Animal studies have shown that the heat sink effect is not significant until the vessel diameter is ≥3 mm [15]. Additional studies have shown a clinically significant increase in tumor recurrence when abutted by a vessel at least 3 mm in diameter [16]. Another concern is damage and thrombosis of surrounding vessels. It has been shown that there is minimal damage and thrombosis to surrounding vessels if the size of those vessels is greater than 3 mm [15]. The implication of these studies is that if a lesion contains a vessel greater than 3 mm, the RFA probe should be placed close to, but not in, the vessel to achieve the best outcome [15, 16]. This placement will move the tumor cells surrounding the vessel into the central ablation zone increasing the likelihood of cell death without significantly increasing damage to the vascular structure.

Other methods to mitigate the heat sink effect have also been studied. One method to overcome the heat sink effect is to occlude the blood supply to the region being ablated with a balloon catheter or gel foam [18]. Since the majority of the blood supply to HCC lesions is

derived from the hepatic artery, temporary occlusion of this artery with a balloon catheter will reduce the heat sink effect and increase the size of the ablative region. Another method of occlusion is embolization of feeding vessels with gel foam. These methods enable the generation of a larger ablative zone. Risks of hepatic artery occlusion and gel foam embolization do exist and should be considered [16].

Injection of NaCl-containing solutions has also been explored to overcome the heat sink effect [16, 19–21]. Pre-treatment with hypertonic saline, 5–36%, results in significantly higher temperatures and a significantly larger ablation zone when compared to no pre-treatment [19, 20]. The NaCl solutions can be injected at any point during the procedure to expand the size of the ablation zone [21].

Transarterial chemoembolization (TACE) combined with RFA is another widely studied technique for overcoming the heat sink phenomenon. Combining these two techniques has multiple benefits that have been shown in both animal and human models [22–24]. First, TACE reduces the amount of conductive cooling by reducing blood flow to the targeted region. The reduction in blood flow increases the size of the central ablation zone, thus increasing the amount of coagulative necrosis. In addition, the amount of tissue that receives sublethal hyperthermia is increased and simultaneously exposed to a chemotherapeutic agent. The synergy is created by increased membrane permeability, intratumoral accumulation of the pharmacologic agent, and increased drug sensitivity of the cells within the transitional zone [5, 6, 8, 24]. Liposomal doxorubicin has been well studied in the setting of liver malignancies and is a good choice of chemotherapeutic agent [22, 24]. The wider ablation margin with increased volume of both the central and transitional zone and high local concentration of chemotherapeutic agent results in destruction of microscopic satellite lesions surrounding the lesion consequently improving local control of the tumor [18, 24].

2.4. Immunologic response to RFA

To adequately discuss the immunologic response to RFA, the basic immunologic response must first be discussed. The immune system is composed of two basic parts, the innate and the adaptive immune systems. Both of these systems work in concert to mount a defense to pathogens, such as viruses and bacteria, and prevent unregulated cell growth. The immune system is able to recognize and eliminate both dangerous self and non-self cells through a system of complex interactions and "danger signals." However, in HCC and other malignancies, the cells evade the immune system through numerous mechanisms [4, 25].

Typically, the first response by the immune system that occurs is by the non-specific or innate immune system. The non-specific immune system is composed of natural killer cells (NK), mast cells, eosinophils, basophils, macrophages, neutrophils, and dendritic cells (DC). These cells are the first to mobilize and produce signals initiating the specific immune response. The specific or adaptive immune system is composed of B and T lymphocytes. With co-stimulation from the innate system, the adaptive system generates a robust and lasting immune response through the formation of antibodies and memory B and T cells. The basic process that must occur to achieve a full immune response is antigen recognition and presentation

by antigen-presenting cells (APCs), subsequent recognition of the antigen by T-cells through interaction with APCs, cellular interaction generating costimulatory signals, and the presence of danger signals [4, 6]. It is important to mention the roles of CD4 or T helper cells (Th) and CD8 T cells or cytotoxic T cells (CTLs). In regards to antitumor immunity, the most important role of CD4 cells is to assist in the activation and proliferation of CD8 T cells. It is currently theorized that a high ratio of CD4:CD8 is important to forming lasting immunity to malignancies because of the role of CD4 cells in stimulating CD8 cells [30]. CD8 T cells have been the focus of antitumor immunity due to their ability to recognize MHC I molecules. MHC I molecules are used to display intracellular antigens on the surface of cells infected by viruses and malignant cells. CD8 cells bind to cells expressing specific MHC I molecule complexes and then destroy the targeted cells through the apoptotic cascade [26].

In contrast to HR where the objective is to completely remove the HCC lesion and occasionally remove local lymphoid tissues, minimally invasive techniques such as RFA leave necrosed tumor cells and their spilled intracellular materials behind. The retained intracellular materials, which were previously invisible to the immune system, can now act as antigens that trigger local and systemic immune responses to HCC. In addition to the release of antigenic material from the remaining necrotic tissues, danger signals, such as DNA, RNA, pro-inflammatory cytokines, uric acid, and heat-shock proteins (HSPs), are released. These damage-associated molecular pattern molecules (DAMPs) are then picked up by DCs and presented to T-cells starting the immune cascade. All of these DAMPs are inflammatory mediators and have the potential to trigger a robust tumor suppressing immune response but are only released by cells undergoing necrosis. Cells, which undergo apoptosis, may actually lead to tolerance if the ratio of apoptosis to necrosis within the lesion becomes too high [4, 25].

One class of DAMP of particular importance to RFA and other thermal ablative techniques is the heat shock protein (HSP) family. HSPs have special roles and are involved in protein folding, cellular signaling, cellular transport, and survival. HSPs are produced within cells in response to thermal injury and play a role as chaperones enabling the refolding of denatured proteins. Additionally, HSPs participate in the initiation of the adaptive immune response by presenting antigens to DCs, modulating DAMP-induced immune stimulation, and function as danger signals [25, 27]. HSPs' ability to chaperone peptides and provide maturation signals to dendritic cells causes the ultimate cross-presentation of antigen to CD8+ T cells. Independent of the adaptive response, HSPs induce local necrosis through stimulation of the innate immune system. HSPs' ability to efficiently stimulate both the innate and adaptive immune system holds great potential; therefore, upregulation of HSPs represents a potential approach to eliminate HCC and other malignancies [28].

In patients treated with RFA, there is a decreased response by CD25+ T-regulatory (Treg) cells [29]. Treg cells or T suppressor cells, a specific subset of T-cells, are responsible for downregulating the immune response. The Treg cell's role is to prevent autoimmune disease and create tolerance to self-antigens. Tregs achieve immunosuppression by downregulating CD4 and CD8 T cells, thus decreasing the tumoricidal immunologic response. In fact, high levels of CD25 T cells are associated with poorer outcomes in patients with malignancy [26]. Thus, patients with HCC can benefit from treatments that decrease the number of Treg cells and subsequent decreased immune tolerance of malignant cells. Indeed, RFA results in decreased

counts of suppressive T cells, but additionally RFA shows increased survival benefit due to improved CD8 T-cell counts. The post ablation increase in CD8 T-cells is strongly associated with decreased recurrence and increased survival in patients with HCC [30]. Patients who will undergo HR or liver transplantation can also benefit from RFA-induced stimulation of CD8 cells. One major issue these patients face long term is disease recurrence. Unitt et al. showed improved survival in patients who demonstrate strong CD4 and CD8 T cell responses after undergoing resection surgery [31]. The response by CD4 and CD8 T cells as well as the increase of specific antibodies has been seen weeks to months following RFA [32].

Most of our knowledge about the immune response to RFA is based on animal models and small human trials. One of the first studies to show a significant immune response to RFA was conducted in 2003. In this animal study, tumors were implanted into rabbits that were then either untouched or treated with RFA. The RFA-treated animals showed at least a threefold increase in specific T-cell infiltration compared to the untreated animals. The treated animals also showed an increase in survival rate. This study suggested that an anticancer immunologic effect could be created through RFA [33]. This study was then further augmented by blocking CTLA-4 with monoclonal antibody at the time of RFA. This strongly enhanced antitumor immunity and provided protection against tumor rechallenge. This demonstrated that a lasting systemic memory response is achievable with combination therapy. Furthermore, a 20-fold increase in specific cytotoxic T-cells (CTLs) was achieved when RFA + blocking antibody was used compared to RFA + control antibody demonstrating that the increased immune response to RFA can be potentiated [34]. Zerbini et al. were the first to demonstrate an increased immunologic response in human subjects. The effect of RFA on 20 patients with HCC was studied and found to have a significant increase in tumor-specific T-cell response. Circulating T and natural killer (NK) cells showed increased activation and expression of specific cytotoxic surface markers. Although an upregulated immune response was demonstrated in these subjects, the effect was not associated with increased protection to HCC relapse [35]. Later, Zerbini and colleagues showed that the immunologic effects post RFA are dependent on maturation of DCs driven by the release of intracellular debris [36]. In a murine urothelial carcinoma model, subtotal RFA was used to induce an immunologic response. In response to subtotal RFA, there was an increase in CD4 and CD8 responses and significant tumor regression with rechallenge [37].

It is clear that numerous benefits of RFA exist and that there is great potential for targeted stimulation of the immune system using RFA in conjunction with immune modulators. Nevertheless, the possibility of causing rapid growth of metastases exists [38–40]. Recent accounts of RFA and other forms of ablation causing growth of distant metastases have been reported. These reports in conjunction with the fact that RFA will induce mediators such as cytokines, including interleukin-6 (IL-6) and factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), hypoxia-induced factor-1α (HIF-1α), and HSPs, all have potential to cause tumor growth locally and distantly [38–42]. It is theorized that damage to the surrounding healthy liver parenchyma and following regeneration is the source of the pro-growth factors. Ahmed et al. [40] showed using a rat model that damaging normal hepatic tissue with RFA will induce distant tumor growth, which is mediated by VEGF and the HGF/c-Met pathway. Interestingly, multiple studies have shown that incomplete ablation of HCC will also promote not only distant growth but also local invasion [41, 43–45].

While increased local and distant growth of tumor cells is a real possibility, there are multiple studies investigating how to mitigate the pro-growth effects created by RFA. Studies have investigated c-Met and VEGF inhibitors to attenuate the tumorigenic effects [38, 40]. Additionally, studies have looked at non-specific anti-inflammatory drugs to mitigate the effects of RFA-induced inflammation on tumorigenesis. Both aspirin and celecoxib have been investigated to prevent tumorigenesis [41, 42]. In animal models, each of these drugs when used in conjunction with RFA reduced local inflammation and subsequent effects on distant tumor cells. Furthermore, it is crucial to note that while there is information about distant tumorigenesis following RFA, clinically RFA has not been shown to worsen survival compared to untreated patients and remains an effective first-line treatment in appropriate patients [46].

3. Cryoablation

3.1. Overview

Cryoablation is a thermal ablative technique that has been used since the nineteenth century. While other thermal ablative techniques add heat to the surrounding tissue, cryoablation removes heat. In its earliest form, a salt and ice solution was applied to breast and skin cancers. This treatment resulted in decreased pain and lesion size. In its current form, cryoablation is performed similarly to other ablative techniques. It can be used in either a percutaneous fashion, with image guidance, or through an open or laparoscopic surgical approach. Cryoablation is used to treat numerous types of cancers, but is most commonly used to treat liver, kidney, lung, prostate, and breast malignancies [47].

Modern cryoablation requires the use of a specialized cryoprobe that is inserted into the targeted lesion (**Figure 1**). Once in the desired location, the probe is rapidly cooled beginning the freeze cycle for a specified length of time. After the freeze cycle is completed, the probe is warmed up to start the thaw cycle. These freeze/thaw cycles are repeated one or more times depending on the lesion and preference of the clinician [47]. The mechanism of cooling relies on the Joule-Thompson effect that describes how a gas that does not work expands (adiabatic expansion) and results in a decrease in temperature [48]. All gases except hydrogen, helium, and neon will decrease in temperature when expanded through the Joule-Thompson process. Commonly used gases for the freeze cycle are nitrogen and argon. One of these gases is pumped into the cryoprobe, and when the gas reaches the distal tip of the probe, the gas is throttled and then allowed to rapidly expand to atmospheric pressure. The result is a rapid decrease in temperature and cooling of the surrounding tissues via conduction. During the freeze cycles, temperatures can reach as low as −160°C, well below the −20 to −40°C required to cause cell death [49]. As mentioned above, helium does not undergo this effect, rather than cooling when rapidly expanded helium will increase the temperature. For this reason, helium is used in the thaw cycle to heat the surrounding tissues [47].

Cryoablation results in direct and indirect cellular injury and death. When the freeze cycle begins, the tissues are cooled and ice starts to form in the extracellular space. Since the formation of ice occurs in the extracellular space before the intracellular space, an osmotic gradient

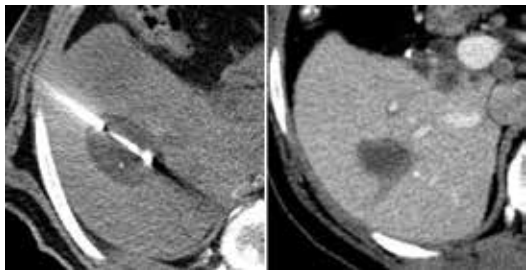

Figure 1. Hepatic cryoablation. A cryoablation needle was advanced into a focal hepatic lesion (left), with a resultant ice ball visible on CT (asterisk). Follow-up imaging demonstrated a focal defect at the site of the previous lesion (right), consistent with a complete ablation.

forms. This gradient pulls free water into the extracellular space increasing the solute concentration and dehydrating the cells. The high concentration of solutes intracellularly results in damage to enzymes and destabilizes membranes of both intracellular organelles and the cell [47]. Intracellular proteins are denatured as well, but return to their original conformation once thawing is completed [50]. Cells at the periphery of the ablation will remain intact and are not immediately killed by cryoablation. These cells will eventually undergo apoptosis that is triggered by damage to organelles [47].

When cells are frozen rapidly, there is no enough time for fluid shifts to occur, and cell death occurs via physical damage to organelles and the cellular membrane from intracellular ice formation. In both cases, pore formation in the membrane occurs, which allows for fluid shifts during the thaw cycle resulting in swelling and rupture [51]. The intracellular fluid shift during the thaw cycle occurs since extracellular ice melts before intracellular ice creating an osmotic gradient into the cell. It is important to note that intracellular ice will continue to grow during the thaw cycle reaching a maximum at −20 to −25°C. This formation of ice during the thaw cycle occurs due to the influx of free water. Additionally, the rate of thawing determines the amount of cellular death. Rapid thawing will decrease the biocidal effect by reducing the amount of intracellular ice formation. A greater degree of cellular death is seen in passive thawing when compared to active thawing. The highest degree of necrosis is seen with repeated freeze thaw cycles [52].

Indirect injury to cells occurs via vascular damage. During the freeze cycle, the endothelium of vessels is damaged, and when thawed, this injury triggers platelet aggregation. This aggregation leads to thrombosis and subsequent ischemia of the tissues [53]. The ischemia is twofold, not only does it lead to cellular death, but it also triggers inflammation. This leads to an influx of neutrophils and macrophages to the ablated zone [54]. The entire process can take months to complete, resulting in a zone of necrosis surrounded by a peripheral band of neutrophils [47].

3.2. Advantages and disadvantages of cryoablation

The one of the best advantages of cryoablation is the ability to monitor the ablation zone in real time. As the ablation proceeds, formation of an ice-ball occurs that is visible on ultrasound (US), magnetic resonance (MR), and CT. This occurs because the water molecules undergo a

phase change and subsequent change in density. For example, during a cryoablation, the ablative zone will become hypoattenuating on CT. The leading edge of the ablation marks 0°C, since this region is where the phase change from liquid to solid is occurring [47].

In contrast to other modalities, such as RFA, each cryoprobe acts independently from the others and can be used simultaneously to tailor the shape of the ablation to the tumor. This is in sharp contrast to ablating with multiple RF probes where only one probe can be active at a time and they must be operated sequentially. Cryoablation additionally offers better pain control when compared to RFA [47]. The cooling of the tissues can create a level of analgesia not offered by hyperthermic ablative techniques. Cryoablation significantly reduces the amount of opioids used in the 24 hours following the procedure leading to shorter hospital stays [55]. In regards to HCC, it has been shown that RFA can induce ischemia-reperfusion injury of the liver resulting in cancer growth. With cryoablation, there is a lower potential for this type of injury decreasing the risk of cancer growth [56]. Cryoablation can also be used in patients who are candidates for RFA. Patients who are candidates are those with tumors <5 cm, single lesions, or multiple lesions <3 cm with a Child-Pugh class A or B liver function [57].

Cryoablation results in a robust inflammatory response following the procedure. This in combination with the fact that the released proteins return to their native conformation produces a large potential to create beneficial antitumor immunologic responses. The large amount of unaltered tumor antigen coupled with a large inflammatory response creates a scenario in which significant numbers of DCs are able to present a large amount of antigen to T cells [47, 50, 54]. The potential immunologic response will be discussed in detail later. This robust inflammatory response also presents a significant disadvantage, cryoshock [47, 57–59].

Cryoshock is a systemic immune response that leads to hypotension, respiratory distress, multiorgan failure, and disseminated intravascular coagulation. Similar reactions are not seen in patients treated with hyperthermic ablations. Cryoshock occurs in up to 1% of patients who undergo hepatic cryotherapy. Of this 1%, up to 18% of patients can die because of cryoshock [57, 58]. Cryoshock is thought to be mediated by the production of cytokine, such as IL-1β, IL-6, and tumor necrosis factor (TNF), from the robust immune response created by cryoablation [47, 57–60]. These are similar to the mediators found in patients with septic shock [59, 65]. Cryoshock typically occurs when large volume liver ablations are attempted [47]. An additional disadvantage of cryoablation is bleeding complications. Typically, these occur when performing large ablations within the liver. Frozen tissues are extremely brittle and may fracture leading to significant bleeding. For this reason, the user must be careful to not torque or reposition the probes once the ablation has started [61]. Although cryoablation has various disadvantages, it has a similar complication rate compared to RFA and remains a relatively safe and effective procedure for the treatment of HCC [57–60, 62].

3.3. Immunologic response to cryoablation

For several decades, the immune response to cryoablation has been known. In the 1970s, antitumor antibodies were first seen in humans following cryoablation [63]. Since then, it has been shown that cryoablation will induce specific anti-tumor cytotoxic effects post ablation. Lymphocytes produced post-ablation show specific affinity for tumor cells when rechallenged,

whereas lymphocytes in patients post HR do not. Reintroduced tumor cells are also rendered less effective by the immunologic response [64].

Cryoablation is proven to create a more robust immunological response than hyperthermic ablative techniques such as RFA and MWA. Higher DC antigen loading due to hypothermic cytotoxicity generates the more robust immunologic response. Increased antigen presentation and subsequent heightened immune response are due to two main factors: increased antigen in the native conformation and less coagulation in the ablation zone [47]. The hypothermic mechanism of cryoablation leads to less regional coagulative effect when compared to hyperthermic ablations. This allows the antigens produced by cryoablation to more readily enter circulation and regional lymph nodes for presentation to DCs. The ability of cryoablation to preserve circulation is beneficial for stimulating the immune system but can be detrimental. The spilling of tumor antigens into circulation and subsequent cytokine release is also believed to be responsible for cryoshock.

Heightened levels of cytokines such as IL-1β, IL-6, TNF-α, and NF-κB are seen after cryoablation [65, 66]. Cryoablation used on hepatic malignancy will increase the levels of these specific cytokines up to 15–25 times more when compared to RFA. Interestingly, Erinjeri et al. found that the changes in WBC count increased linearly with ablation size but the levels of IL-6 did not [65], suggesting that larger ablative zones could trigger higher tumor-specific immune responses without added increased risk for cryoshock. Additionally, this group found that the predominant cytokines that are released post cryoablation, IL-6 and IL-10, stimulate a Th2 response.

According to the Th1/Th2 model, each subset triggers a different type of immunity. Th1 triggers cytotoxic lymphocytes and cellular immunity, whereas Th2 stimulates B-cells and antibody production. Signals that stimulate the Th1 response include IL-2, IL-12, IFN-γ, and TNF, and the cytokines that trigger the Th2 response include IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 [65, 67]. In addition, IL-6, which activates NF-κB and STAT3, has been implicated in hepatic regeneration and increased tumor regeneration post ablation. Regeneration is mediated by NF-κb and STAT3, both of which are activated by IL-6 [65]. Since, both IL-6 and IL-10 are increased after not only cryoablation but also hyperthermic ablation, they are potential targets for adjuvant immunologic therapy. Decreasing IL-6 could potentially increase the Th1 response (cellular-mediated tumor immunity) and decrease the growth of primary and metastatic hepatic malignancy post ablation [68].

Immune checkpoint inhibitor therapy, a new oncologic therapy that uses monoclonal antibody to target and block the T cell surface receptor CTLA-4, has potential use in combination therapy with cryoablation. The function of CTLA-4 is to inhibit self-reactive T-cells in order to prevent autoimmune diseases. However, in the case of cancer, it is beneficial for T cells to be able to recognize specific "self" cells. It has been shown that blockade of CTLA-4 will increase the CD8 T cell response as well as CD4 T cell memory when used as a monotherapy or combination immune therapy [69, 70]. Phase 3 trials using ipilimumab, a CTLA-4 blocking monoclonal antibody, have showed improved recurrence-free survival when used as an adjuvant treatment in patients with high-risk melanoma [71]. Combination cryotherapy with CTLA-4 blockade has been studied in prostate cancer. While cryoablation alone has not been

shown to mediate the rejection of metastatic lesions, when combined with CTLA-4 blockade, it can mediate rejection of metastatic lesions and prevent disease recurrence [72].

While cryoablation has been strongly shown to activate the immune system, the opposite has also been seen. Multiple animal models have shown susceptibility to rechallenge and increased metastasis post ablation [73–75]. A possible explanation for these results is variation in the technical factors of cryoablation. Differing animal models, methods of freezing, length, number of freeze-thaw cycles, differences in minimum temperature achieved as well as differing ablation zone size and position all contribute to different clinical outcome and immunologic stimulation or anergy [73]. Sabel et al. established that variation in the technical parameters of cryoablation indeed affect the ratio of apoptosis to necrosis and subsequent immune response. Sabel et al. investigated the rate of freezing in an animal model using either a low or high rate of freezing. They found that a high rate of freezing induced a higher amount of necrosis when compared to a low rate of freezing. The high rate induced more danger signals stimulating a strong anti-tumor response [73]. A high ratio of apoptosis to necrosis has been shown to downregulate the immunologic response and even induce anergy [76]. When apoptotic cells are presented to DCs, a lower amount of TNF-α, IL-1β, IL-8, IL-10, IL-12, and granulocyte macrophage colony-stimulating factor (GM-CSF) produce inhibitory effects on these cells [77, 78]. The ablation zone size and percentage of tumor encompassed may play a role in the immunologic response. An experiment conducted in a murine metastatic liver tumor model demonstrated that smaller volume ablations show a significant decrease in metastasis [79].

4. Microwave ablation

4.1. Overview

Microwave ablation (MWA) is a hyperthermic ablative technique that is similar in many ways to RFA. MWA was introduced in the 1980s and 1990s and showed potential, but suffered from problems controlling the emitted field. There was a relatively high complication rate with MWA, thus RFA became the dominant ablative technique [80, 81]. While the early MWA systems had higher complication rates, since then newer designs have significantly decreased the complication rates. Recent retrospective and prospective studies have proven the efficacy and safety of MWA for not only HCC lesions but also other hepatic lesions [82–85].

From a procedural point of view, MWA and RFA are performed similarly under image guidance. The operator guides a MWA antenna toward the targeted lesion using their favored imaging modality (**Figure 2**). Unlike RFA, MWA does not require the use of grounding pads to establish an electrical circuit. MWA uses dielectric hysteresis to produce heat. An oscillating field, typically 900–2500 MHZ, is applied forcing polar molecules (such as water) to continuously move and realign in the field creating kinetic energy and ultimately raising the temperature of the tissue. Microwaves are able to propagate through a variety of tissues, even those with low electrical conductivity, high impedance, or low thermal conductivity. This makes MWA more versatile [86].

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4.2. Advantages and disadvantages of MWA

MWA has the distinct advantage of being able to penetrate through high impedance tissues, meaning that even if charred or desiccated tissues build up near the probe, the field is able to penetrate and continue enlarging the ablation zone. Since MWA does not rely on conduction of tissue, heat is able to penetrate tissues with a high impedance such as lung or bone [86].

Multiple MWA antennas are able to be used synergistically to enlarge the ablation zone, achieve higher temperatures, or concomitantly ablate multiple lesions [87]. While RFA using multiple probes requires the probes to be used in series, MWA with multiple antennas can be used simultaneously with one power source. Due to the properties of MWA, there is future potential use ablating larger lesions than is currently possible.

The peak temperatures achieved in the central zone can readily exceed 100°C. The ability to achieve higher temperatures and use multiple probes simultaneously means shorter treatment times and larger area of coagulative necrosis and lethal hyperthermia. Higher temperatures and larger central zone lessen the effect of nearby heat sinks. It has been shown that large vessels <10 mm in size will not affect the ablation, making it possible to ablate lesions in regions that are not possible with RFA [88].

While MWA has many advantages, its ability to deliver a high amount of energy comes with several trade-offs. Coaxial cables have excellent properties for this application and are thus used to connect the antenna to the microwave generator. However, the coaxial cables used have a large diameter in order to avoid dangerous cable overheating. Larger diameter decreases the risk of overheating but becomes cumbersome and inflexible leading to difficulties while manipulating the antennas and performing the procedure [89]. The microwave antennas are likewise made using coaxial cable and also suffer from the same problem. In order for the antenna to handle higher power levels, the diameter must be increased or an active cooling system needs to be employed [86, 89].

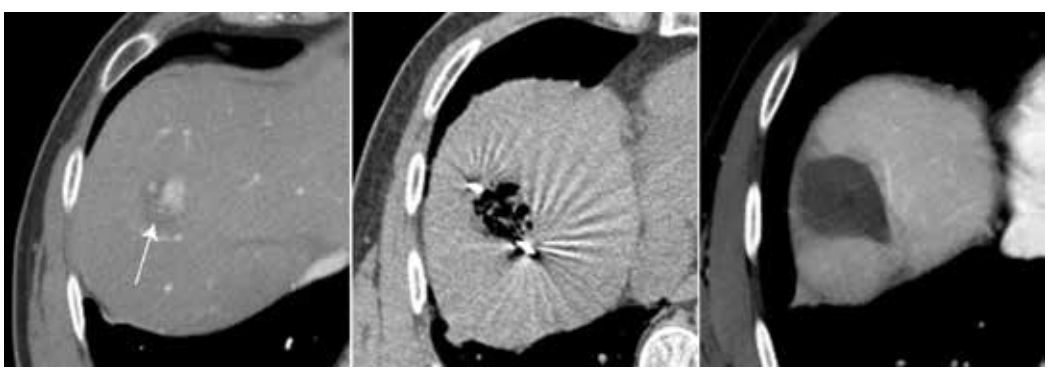

Figure 2. Hepatic microwave ablation. Pre-procedure imaging demonstrates a focal HCC lesion at the hepatic dome (left, arrow). Two microwave needles were advanced into the lesion, with gas bubbles developing during the ablation (middle). Follow-up imaging demonstrates a focal defect without enhancing viable tissue consistent with a complete ablation (right).

Active cooling systems have helped eliminate several problems. They allow a smaller diameter antenna to handle higher power and eliminate the risk of ablating healthy tissues along the proximal antenna tract, increase the size of the ablation, increase the amount of power delivered, and can prevent the probe from backing out. Various cooling methods have been employed from chilled saline to cooling with compressed gas utilizing the Joule-Thomson phenomenon [86]. Some newer probes that use the Joule-Thompson phenomenon are able to be locked into place by freezing the antenna tract to prevent it from moving or backing out.

Since the primary advantage of MWA is the ability to deliver, a significant amount of power safety concerns arises. With MWA, it is harder to predict the size of the ablation zone, which can lead to damage of surrounding structures. The shape of the ablation zone produced can be relatively thin and long increasing this risk. While problems with MWA exist, currently these issues should not limit its use ablating HCC and other lesions in the liver. MWA has been proven effective and comparatively safe to RFA when measuring complication rates [82–85].

4.3. Immunologic response to MWA

The immunologic response to MWA is less well characterized compared to other methods of ablation, such as RFA and cryoablation. The vast majority of the research regarding the immune response and immunologic stimulation has been studied with either RFA or cryoablation. Recently specific immunologic mechanisms and the effects following MWA have been studied in more detail. It has been assumed that the immunologic response to MWA is similar to the mechanism and response to RFA [90, 91]. Currently, our knowledge about the immune response to MWA comes from both animal and clinical trials of various tumor types from breast to hepatic carcinomas [92–94].

In regards to patient management and future treatment, the goal of therapy is to generate a lasting immune response that results in regression of distant lesions and generate protection from disease recurrence. As detailed in previous sections, the aim of treatment is to generate specific cytokines triggering the Th1 response and activating the cellular immune system. Similar to other ablative techniques, MWA alone is not powerful enough to trigger the desired immune response, but holds potential with combination therapy [90, 91].

The specific immune response to MWA in patients with HCC has been analyzed by Zhang et al. In their study, 45 patients with HCC treated with MWA had peripheral blood analysis following treatment. The results showed significant increases in IL-12 and decreases in IL-4 and IL-10 [91]. These results are promising since IL-12 is involved in the differentiation of Th1 cells and generation of cellular immunity. Furthermore, patients showed decreased levels of IL-4 and IL-10, which are involved in activation of humoral immunity. While MWA alone is not enough to create a significantly different clinical response, the cytokine profile produced is advantageous.

Various combination therapies have been studied ranging from OK-432, immunotherapy, GM-CSF, and CTLA-4 blockade [92–94]. OK-432, also known as picibanil, is a low virulence mixture of *Streptococcus pyogenes* that has been used as an antitumor agent since 1975 [95].

OK-432 is able to induce pro-inflammatory cytokines and activate the T-cell–mediated immunity. Li et al. demonstrated that MWA and OK-432 used in combination resulted in prolonged survival and a strong immunologic response to rechallenge in a murine model of breast cancer. The results showed that a dominant Th1 response is generated. The cytokines IL-12, IL-2, and IFN-γ were significantly increased with no effect on Th2-type cytokines. Additionally, immunohistochemical analysis showed that a predominance of CD8+ T cells infiltrating the treated tumors.

Immunotherapy combined with MWA for the treatment of HCC has been investigated in both phase I and phase II trials [93, 96]. This combination was shown to increase the absolute number of circulating lymphocytes. When analyzed for specific subgroups, patients showed increased levels of cytotoxic subsets of T cells and decreased suppressive subsets. Additionally, patients treated with immunotherapy had significantly improved liver function. However, the disease-free survival and overall survival rate were not significantly improved.

CTLA-4 blockade holds great promise when combined with cryoablation, but could also be used in with MWA. CTLA-4 blocking antibodies and GM-CSF combined with MWA were shown to induce tumor-specific cellular immune response in a murine model [94]. The combination of the three resulted in a 90% rejection upon tumor rechallenge and 50% of the animals treated showed distant tumor regression. Since both of these drugs are currently available for human use, this combination represents one that could be clinically used today.

5. Conclusion

Minimally invasive thermal-based therapy has become a reliable method for the treatment of HCC. Many advances in ablative therapy have occurred since their initial implementation ranging from design to technical implementation. The most promising of these advances is combination therapies that create a tumor-specific immunologic response. Combination therapy has shown great promise in the treatment and prevention of not only HCC, but also other malignancies. There is much more to learn about the immunologic reaction to ablative therapy creating an exciting time of investigation and discovery.

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Chapter 8

Emerging Targeted Therapies for Treatment of Hepatocellular Carcinoma (HCC)

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Additional information is available at the end of the chapter

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Abstract

Hepatocellular carcinoma (HCC) has dismal diagnosis due to the presence of underlying cirrhosis, late diagnosis, and limited treatment options. Surgery or liver transplantation is restricted to those with small tumours or well-compensated liver diseases. Despite advances in early screening and diagnosis of HCC, survival of patients has not improved greatly. Furthermore, treatment options for advanced HCC are restricted to best supportive care. Currently, sorafenib is the only drug approved for the treatment of advanced HCC patients as well as for those not suitable for transarterial chemoembolization (TACE). Therefore, there is an urgent need to develop new agents for treatment. Hepatocarcinogenesis is a complex multistep process that involves deregulation of various signalling pathways. Thus, there is no dominant molecular mechanism in HCC and understanding of these pathways provides an opportunity for development of potential therapeutic agents in an effort to reverse, prevent or delay tumourigenesis. This review will summarise the significance of these pathways in HCC and discuss the therapeutic benefits or drawbacks of the potential target agents against these pathways especially those that have been part of clinical trials.

Keywords: hepatocellular carcinoma, targeted therapy, sorafenib, signalling pathways, immunotherapeutics

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of liver cancer and is the second biggest cause of cancer-related deaths world-wide. The incidence of HCC is increasing all over the world but the highest rates of HCC are reported in South-East Asia with the leading rate of mortality occurring in China [1]. The risk factors for HCC are well

defined, such as hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, alcohol consumption, and non-alcoholic steatohepatitis (NASH) [2].

The main reasons for the high rate of mortality are lack of diagnostic methods and limited treatment options for patients with advanced HCC. Surveillance programmes to identify patients with early HCC, such as by ultrasound sound screening and by serum alpha fetoprotein (AFP) levels, are not well implemented. Additionally, AFP levels are also dysregulated in benign liver diseases [3]. Some of the treatment options for HCC patients include surgical resection, liver transplantation (LTx), radiofrequency ablation (RFA), transarterial chemoembolization (TACE) and sorafenib. Surgery has a 5-year survival rate of 70% but unfortunately at the time of diagnosis, only 10–30% of patients are suitable for this option. The biggest risk post-surgery is that of recurrence. The 5-year recurrence rate in patients with early HCC is about 68% after surgery [4]. LTx is suggested to HCC patients with tumours within the Milan criteria (a single lesion ≤5 cm, or up to three lesions ≤3 cm each) and is associated with a 5-year overall survival (OS) rate of 75%. However, the limitation of LTx is shortage of organ donation [5]. RFA is another option for patients with early HCC (<3 cm) and its survival benefits are comparable to those with surgical resection. However, high costs of RFA and complications involving peritoneal bleeding hinder its use [6]. TACE is the standard of care for intermediate HCC with preserved liver function, and with no signs of macrovascular invasion or extrahepatic spread. It has reported a median survival of 34 months and a survival benefit at 1, 3, 5 and 7 year as 82%, 47%, 26% and 16%, respectively. However, TACE is a heterogeneous operating technique with variation in efficacy depending on the choice of chemotherapeutic agents used [7]. For advanced HCC patients, sorafenib is the only drug approved by the

Trial name and no.	Drug	Target	Ref
SHARP, NCT00105443	Sorafenib *versus* placebo	VEGFR, PDGFR, Raf	[8]
Asia Pacific, NCT00492752	Sorafenib *versus* placebo	VEGFR, PDGFR, Raf	[15]
STORM, NCT00692770	Sorafenib *versus* placebo	VEGFR, PDGFR, Raf	[17]
REACH, NCT01140347	Ramucirumab *versus* placebo	VEGFR-2	[23]
--, NCT00699374	Sunitinib *versus* sorafenib	VEGFR, PDGFR	[26]
LIGHT, NCT01009593	Linifanib *versus* sorafenib	VEGFR and PDGFR	[28]
RESOURCE, NCT01774344	Regorafenib *versus* placebo	VEGFR1-3, PDGFR-β, and FGFR-1	[30]
EVOLVE-1, NCT01035229	Everolimus *versus* placebo	mTOR	[39]
SILVER, NCT00355862	Sirolimus *versus* placebo	mTOR	[43]
METIV-HCC, NCT01755767	Tivantinib *versus* placebo	c-MET	[47]
JET-HCC, NCT02029157	Tivantinib *versus* placebo	c-MET	[47]
CELESTIAL, NCT01908426	Cabozantinib *versus* placebo	c-MET and VEGFR, PDGFR	[48]

--: no name assigned; NCT: ClinicalTrials.gov number; VEGFR: vascular endothelial growth factor receptor; PDGFR: platelet-derived growth factor receptor; FGFR: fibroblast growth factor receptor; mTOR: mammalian target of rapamycin.

Table 1. Phase III clinical trials of molecular targets in HCC.

US Food and Drug Administration (FDA) for treatment and although it improves survival compared to placebo in clinical trials it suffers from adverse side-effects and high costs [8]. Unfortunately, with a majority of patients still diagnosed at late stage and with clinical phase III trials failing to improve survival benefits in intermediate and/or advanced HCC, new molecular therapeutics are urgently needed to address the dismal prognosis of HCC. One approach is to identify molecular targets from the several signalling pathways that are dysregulated in HCC. Several phase III trials have been completed to identify potential molecular targets in HCC (**Table 1**).

2. Vascular endothelial growth factor (VEGF) receptor signalling

Angiogenesis is a critical step for tumour growth and metastasis. With HCC being a highly vascular tumour, controlling tumour-associated angiogenesis offers a promising approach to inhibiting tumour progression.

VEGF is the most well documented growth factor in angiogenesis. It exerts its effect by binding to its receptors, VEGF receptor 1 (VEGF-R1), VEGF-R2, and VEGF-R3, present on endothelial cells. VEGF secreted by tumour cells bind to its receptors and results in activation of signal transduction pathways promoting cell migration, proliferation, and survival of cancer cells leading to angiogenesis. VEGF overexpression is possibly induced by the hypoxic tumour environment, activation by epidermal growth factor (EGF) receptor (EGFR) and cyloonxygenase-2 signalling. Increased levels of VEGF, VEGFRs have been reported in HCC cell lines, tissue and serum of HCC patients. High levels of VEGF in HCC patients has been associated to poor OS and disease-free survival [9], vascular invasion [10] and portal vein emboli [11]. Additionally, Guo et al. [12] and colleagues reported poor prognosis for HCC patients with increased serum VEGF following TACE. VEGF is also more commonly expressed in HCV-associated HCC than in HBV-associated HCC providing clinical implications for different population of HCCs.

Other growth factors stimulating angiogenesis include fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptor (PDGFR). Overexpression of either of these growth factors has also been associated to poor survival. There are four types of FGFRs (FGFR1, 2, 3, 4) and the PDGFR consist of PDGFRα and PDGFRβ [13, 14].

Sorafenib is currently the only drug approved for treatment of advanced HCC patients who cannot undergo TACE treatment. It is an orally active anti-angiogenic multi-kinase inhibitor. Several clinical studies have reported promising results. In the randomised phase III SHARP trial (ClinicalTrials.gov number NCT00105443), 400 mg of sorafenib twice daily, significantly increased the OS of advanced HCC patients (7.9 months *versus* 10.7 months) and the time to progression (TTP) (2.8 months *versus* 5.5 months) compared to the placebo group [8]. Similarly in another phase III Asia Pacific trial (NCT00492752), sorafenib increased the OS and TTP from 4.2 months to 6.5 months and from 1.4 months to 2.8 months, respectively [15]. The difference in the OS and TTP results in both studies could be due to patient HCC aetiology. The Asia Pacific trail had more HBV-associated HCC compared to the SHARP trial (73% *versus* 12%).

The use of sorafenib as an adjuvant after surgery or TACE remains doubtful. In a small retrospective study with 36 HCC patients, 12 patients received sorafenib post-surgery and the remaining 24 patients had surgery only. The group of patients who received sorafenib post-surgery had a significantly longer OS (37 months *versus* 30 months) and TTP (29 months *versus* 22 months) [16]. However, in the phase III placebo-controlled study (STORM, NCT00692770), which recruited 1602 patients from 28 countries, sorafenib as an adjuvant treatment after surgery/local ablation, did not affect time to recurrence or OS [17]. Similar findings were reported in the SPACE trial (NCT00855218). In this phase II trial Lencioni et al. [18] tested the efficacy of doxorubicin-eluting beads (DEB)-TACE plus sorafenib *versus* sorafenib in patients with intermediate HCC. The authors did not report a significant improvement in TTP following addition of sorafenib to DEB-TACE.

Bevacizumab is an anti-VEGF monoclonal antibody that has demonstrated improved efficacy in patients with unresectable HCC. Treatment with bevacizumab at 5–10 mg/kg produced partial response (PR) in 14% and disease control rates (DCR) in 56% of patients. A phase II trial of bevacizumab with capecitabine and oxaliplatin (chemotherapeutic drugs) also showed encouraging results with a median progression-free survival (PFS) of 6.8 months, and a median OS of 9.8 months. Twenty three patients had stable disease with overall 77.5% disease control rate and eight patients produced (PR) [19]. Hsu et al. [20] investigated the combination of bevacizumab plus capecitabine in a phase II study yielding median PFS and OS of 2.7 and 5.9 months, respectively.

Ramucirumab is another example of monoclonal antibody targeting VEGFR-2. The above mentioned bevacizumab targets the proangiogenic factor VEGF while ramucirumab blocks the receptor. In a phase II study involving advanced HCC patients, ramucirumab monotherapy yielded a disease control rate (DCR) of 50%, PFS of 4.0 months and OS of 12 months [21]. The promising results from this study lead to a phase III trial (REACH, NCT01140347) of ramucirumab monotherapy in advanced HCC patients (post-sorafenib). Although ramucirumab did not improve OS, interestingly in a sub-group of HCC patients with AFP base line levels ≥400 ng/mL, ramucirumab significantly enhanced the OS [22].

Sunitinib is an orally administered multi-kinase inhibitor with activity against various kinases including VEGFR and PDGFR. It has been approved for treatment of renal cell carcinoma (RCC), and imatinib-resistant gastrointestinal stromal tumours (GIST). However, it is not considered for HCC patients due to its high toxicity. In two phase II studies of sunitinib, 50 mg daily of sunitinib orally, 4 weeks on and 2 weeks off, both Barone et al. [23] and Faivre et al. [24] reported high toxicity. Barone et al. [23] observed treatment-related deaths in 18% of patients and with PR in 12% of patients. Median TTP was 2.8 months and median OS was 5.8 months. Faivre et al. [24] reported 10% deaths related to treatment and 80% patients experienced grade 3/4 adverse effects because of which the study could not proceed to the second phase and was terminated [23, 24]. Similarly, a phase III trial (NCT00699374) comparing sunitinib to sorafenib was discontinued. Patients were administered 37.5 mg of sunitinib once daily or 400 mg of sorafenib twice a day but a majority of patients experienced adverse effects such as thrombocytopenia and neutropenia. Additionally, sunitinib did not show a better OS than sorafenib [25].

Linifanib is a multikinase inhibitor targeting VEGFR and PDGFR. In a phase II trial involving 44 HCC patients with unresectable or metastatic HCC, linifanib yielded a median OS of 9.7 months (compared to 10.4 months in patients with Child-Pugh class A hepatic function) [26]. In an open-label phase III trial (LIGHT, NCT01009593), Cainap et al. [27] compared linifanib with sorafenib treatment in advanced HCC. Both drugs had similar OS with 9.1 months for linifanib and 9.8 months for sorafenib. The median TTP was found to be 5.4 months and 4.0 months for linifanib and sorafenib respectively. However, linifanib caused more adverse side effects than sorafenib, implying sorafenib could be more safe than linifanib.

Regorafenib is a novel diphenylurea multikinase inhibiting VEGFR1-3, PDGFR-β, and FGFR-1. It has been approved for treatment of metastatic colorectal cancer after failure of oxaliplatin and irinotecan-based systemic chemotherapy and has also been approved for treatment of metastatic gastrointestinal stroma tumours after failure of imatinib and sunitinib. This year regorafenib was approved by the FDA as a second-line treatment for HCC. HCC patients not responding to sorafenib now have an option of FDA-approved regorafenib as a second line of treatment. This makes regorafenib the first FDA approved drug for treatment of liver cancer in almost a decade. In a small phase II study involving 36 advanced HCC patients who had progressed following sorafenib, regorafenib at 160 mg once daily in cycles of 3 weeks yielded a median TTP of 4.3 months median OS of 13.8 months. The side effects of regorafenib appeared similar to that of sorafenib such as fatigue, diarrhoea, hypertension and, hand–foot skin reaction [28]. A phase III trial of regorafenib (RESOURCE, NCT01774344) involving 573 patients from 21 countries evaluated the efficacy and safety of regorafenib in HCC patients and observed disease progression after systemic first-line treatment with sorafenib. Regorafenib treatment resulted in a survival benefit of 2.8 months compared to placebo (10.6 months *versus* 7.8 months). The median PSF for patients taking regorafenib was 3.1 months compared to 1.5 months for patients taking placebo. The overall response rate was 11% compared to 4% of patients taking placebo [29]. Following these promising results from the RESOURCE trail, regorafenib was approved by the FDA in April 2017 for the treatment of HCC patients who have previously been treated with sorafenib.

3. RAF/MEK/ERK pathway

The mitogen-activated protein kinase (MAPK) cascade consists of serine/threonine kinases, which converts extracellular molecules such as growth factors, hormones, and differentiation factors, into intracellular signals for regulating several cellular processes including proliferation, apoptosis and migration. The four core proteins kinases of this pathway include, Ras, Raf, MEK and ERK. The pathway is activated by binding of ligand to receptor tyrosine kinases (RTK). In the nucleus, phosphorylation of these four protein kinases regulates gene transcription. Around 58% of HCC cases have activated MAPK pathway with Ras, MEK, ERK and MAPK up regulated in 33%, 40%, 50% and 50% of HCC patients, respectively [30]. This pathway has also been shown to be activated by hepatitis virus infections. Dysregulation of this pathway by hepatitis B virus X protein has contributed to loss of function of the tumour

suppressor p53 [31]. HCV infection has led to anti-apoptotic effect also following activation by Ras/Raf/Mek/Erk signalling [32].

Selumetinib is an oral MEK inhibitor. In a small phase II study (NCT00604721) involving 19 HCC patients with advanced HCC, selumetinib was given at a dose of 100 mg twice per day but the study was terminated at interim analysis because there was no response and the TTP was only 8 weeks. However western blot of biopsy samples taken pre and post treatment showed phosphorylation of MEK1/2, and ERK1/2, suggesting failure of selumetinib was not due to lack of target inhibition [33]. A recent phase I study (NCT01029418) looked into the safety, maximum tolerated dose (MTD), and tolerability of selumetinib in combination with sorafenib in 27 Asian patients with advanced HCC. The MTD of selumetinib was at 75 mg daily with sorafenib at 400 mg twice daily. Common treatment-related adverse events included diarrhoea, rash, and hypertension, fatigue, anorexia and hand-foot and mouth disease. Seven patients had a PR and stable disease for more than 6 months. The OS was 14.4 months. Due to the acceptable adverse events, this combination of selumetinib and sorafenib deserves further evaluation [34].

Another MEK inhibitor, refametinib, was evaluated in a phase II study (NCT01204177) in combination with sorafenib in 95 patients with unresectable HCC. Patients received twice-daily refametinib at 50 mg plus twice-daily sorafenib at 200 mg (morning)/400 mg (evening), with dose escalation to sorafenib 400 mg twice daily after cycle 2. The TTP was 122 days and OS was 290 days. Interestingly, the best responders to the combination treatment were those harbouring Ras mutation. A recently completed proof of concept phase II trial (NCT01915602) of refametinib in combination with sorafenib in Ras mutant HCC has recently been completed with results expected soon. Given that Ras mutations are only observed in 3–5% of HCC patients, this study raises questions about feasibility and costs of screening large cohort of patients to identify a small sub-group with particular mutations.

4. Mammalian target of rapamycin (mTOR) signalling pathway

This pathway is a critical regulator of numerous physiological processes and also plays a pivotal role in cell proliferation and metastasis of transformed human cancers including HCC. It is upregulated in around 40% of HCC and has been associated to poor prognosis and early recurrence independent of underlying liver aetiology [35]. Two mTOR inhibitors have been studied in clinical trials.

Preclinical studies have shown everolimus (taken orally) to dose-dependently inhibit tumour growth in patient-derived xenograft models of advanced HCC [36]. In a phase I/II study (NCT00516165) in advanced HCC patients, Zhu et al. [37] reported daily dose of 10 mg per day to be well tolerated in 28 patients producing a medium PFS and OS of 3.8 months and 8.4 months respectively. The subsequent phase II study involving 28 patients with prior systemic therapy with daily dose of 10 mg could not be completed as two patients remained progression free for 24 weeks. Although everolimus was well tolerated this study had some limitations including small sample size and lack of randomised control. The efficacy of

everolimus was next investigated in advanced HCC patients who did not respond to sorafenib. In a phase III, randomised, double-blind study (EVOLVE-1, NCT01035229) everolimus did not show improvement in OS (7.6 months with everolimus, 7.3 months with placebo). In a separate phase II study (NCT01005199), patients with advanced HCC were compared to those administered sorafenib alone (800 mg) or with everolimus (5 mg) [38]. The results were not encouraging and combination of sorafenib with everolimus did not improve efficacy compared to sorafenib alone with median PFS (6.6 months *versus* 5.7 months), TTP (7.6 months *versus* 6.3 months), and OS (10 months *versus* 12 months) were similar in the Sorafenib alone group *versus* sorafenib + everolimus, respectively. However, loss of tuberous sclerosis complex 2 (TSC2) in HCC has been reported to be predictive of response to everolimus in HCC patients [39]. Immuohistochemical analysis of HCC samples collected in the EVOLVE-1 clinical trial (NCT01035229) had no detection of TSC2 and longer OS than compared to placebo. A larger study is needed to validate the potential of everolimus before it can be used to stratify HCC patients for response to everolimus.

Another mTOR inhibitor, temsirolimus (taken intravenously) has not improved survival either alone or in combination with either sorafenib [40] or bevacizumab [41]. In a recently concluded phase III study (SILVER, NCT00355862), another mTOR inhibitor, sirolimus, did not improve recurrence-free survival (RFS) or OS beyond 5 years in Ltx recipients with HCC but it did improve RFS and OS within 3–5 years. This may suggest the potential use of sirolimus for selection of immunosuppression in LTx recipients with HCC [42].

5. c-MET inhibitors

c-Met is a proto-oncogene that encodes the receptor, MET, for the ligand of hepatocyte growth factor (HGF). MET is a tyrosine kinase receptor regulating metastatic progression. Binding of MET to HGF activates the RAS-MAPK and PI3K-AKT signalling pathways leading to tumour development and metastasis. In HCC, c-MET protein is overexpressed in 70% of HCC and has been associated to poor prognosis [43].

Foretinib was the first c-MET inhibitor of broad spectrum, including c-Met and VEGFR, to be tested in clinical trials. In a phase I/II study (NCT00920192) involving patients with advanced HCC, the median TTP was 4.2 months and the OS was 15.7 months. Its toxicity profile was also acceptable with the most adverse events including hypertension and anorexia. Baseline plasma levels of Interleukin 6 (IL6) and Interleukin 8 (IL8) were identified as independent predictors of OS by multivariate analysis. A larger randomised study is needed to warrant the effects of foretinib [44].

Tivantinib is a selective oral inhibitor of c-MET. In a randomised, placebo controlled phase II study (NCT00988741), advanced HCC patients were administered 240 mg daily resulting in a small improvement in TTP (1.4 months *versus* 1.6 months) compared to the placebo group. Additionally, HCC tumours expressing high levels of c-MET protein, as judged by immunohistochemical analysis, demonstrated an improved OS (7.2 months *versus* 3.8 months) and longer TTP (2.7 months *versus* 1.4 months) compared to placebo. There was no difference

in OS and TTP in HCC patients with c-MET protein expression between tivantinib and placebo. These results suggest the potential of c-met protein expression to select HCC patients who may benefit from tivantinib [45]. However, surprisingly, two large randomised double-blind placebo-controlled phase III trials i.e. METIV-HCC (NCT01755767) and JET-HCC (NCT02029157), have both failed to demonstrate improved OS in advanced HCC patients with high c-met protein expression [46].

Cabozantinib is also an oral inhibitor of c-MET, VEGFR and PDGFR. *In vitro* and *in vivo* studies have demonstrated its reduced invasive and migratory properties in HCC. A phase II randomised trial is on-going to investigate the efficacy to cabozantinib in solid tumours. A phase III, randomised, double-blind, controlled trial is underway to evaluate the efficacy of cabozantinib *versus* placebo as a second-line treatment for advanced HCC who have received prior sorafenib (CELESTIAL, NCT01908426) [47].

6. Other potential therapeutic targets in HCC

6.1. Wnt/β-catenin signalling

The Wnt/β-catenin signalling plays a pivotal role in a host of physiological and pathophysiological processes such as embryonic development, cell proliferation, regeneration, angiogenesis and cancer [48]. It is also an important player in maintaining liver health, but it is found to be dysregulated in HCC with mutation in β-catenin observed in about 40–70% of HCC cases, proving to be a potential important target of therapy.

At physiological levels β-catenin is regulated by a destruction complex consisting of adenomatous polyposis coli (APC)/Axin/glycogen synthase kinase 3b (GSK3β), and casein kinase 1 (CK1) which phosphorylates β-catenin at Ser33, Ser37, Thr41, and Ser45 residues located in exon 3. The phosphorylated β-catenin is polyubiquitinated by β-transducin repeat containing protein (β-TrCP) and degraded by the proteasome. However, wnt signalling is activated upon binding of the wnt to one of the frizzled (FZD) family members and to low- density lipoprotein receptor-related protein 5 (LRP5) or LRP6, resulting in the inhibition of β-catenin degradation. The accumulated cytoplasmic β-catenin translocates to the nucleus where it forms a complex with T-cell factor (TCF)/lymphoid, displacing the transcriptional inhibitor Groucho, and the β-catenin-TCF complex enhances transcription of target genes that are implicated in cancer development for example, c-Myc and cyclin D1.

Nuclear β-catenin accumulation has been found to be associated to tumour progression and poor prognosis. Cytoplasmic β-catenin accumulation has been reported in HCCs larger than 5 cm in diameter and with reduced disease-free survival. Dysregulation of the wnt/β-catenin signalling has also shown to regulate angiogenesis and metastasis [49]. Aberrant activation of wnt signalling has also resulted from deregulation of other components of the pathway e.g. up regulation of wnt genes (Wnt3, Wnt4 and Wnt5A) and FZD (FZD3, FZD6 and FZD7) in about 60–90% of HCCs with more than 5% occurring in peritumours, implying that their expression could be an early event in hepatocarcinogenesis [50].

Disruption of β-catenin and TCF association in the nucleus by two fungal-derived compounds, PKF115-584 and CGC049090, has shown dose-dependent cytotoxicity against HCC cells and 10 times reduced toxicity in normal hepatocytes [51]. The disruption reduced expression of wnt/β-catenin target genes (c-Myc, cyclin D1, survivin) and inhibited *in vivo* tumour growth [52]. For reasons yet to be delineated, the presence of EpCAM, hepatic stem cell marker and a direct target of the wnt/β-catenin pathway, sensitised HCC cells to these antagonists [53]. Together these results suggest that EpCAM expression may facilitate HCC prognosis by effective stratification of HCC patients responsive to wnt/β-catenin signalling antagonists.

Recently, two FDA-approved drugs have been identified to antagonise wnt/β-catenin pathway by differing mechanisms. First, pyrvinium was identified in a chemical screen for small molecules. It binds to CK1 potentiating its activity and leading to stabilisation of the destruction complex resulting in degradation of cytoplasmic and nuclear levels of β-catenin [54]. Recently, Pimozide, an antipsychotic drug, has been shown to inhibit cell proliferation and apoptosis in HCC cell lines by reducing EpCAM and β-catenin [55]. The specific role of these inhibitors has yet to be completely elucidated.

Another class of compounds regulate the wnt/β-catenin pathway by inhibiting tankyrases (TNK1 and TNK2). TNKs destabilise Axin leading to β-catenin stabilisation. Thus, inhibition of TNKs prolongs half-life of Axin preventing β-catenin accumulation. These compounds include XAV939 and WXL-8 and also reduce tumourigenicity *in vivo* [56].

Another therapeutic strategy to regulate the wnt/β-catenin signalling is to block the interaction between wnt ligands and FZD receptors. This has been achieved with monoclonal antibodies or using recombinant soluble fragment of FZD (sFZD). A monoclonal antibody, OMP-18R5, developed using the extracellular domain of FZD7, binds to five FZD receptors and blocks wnt signalling. It inhibits *in vivo* tumour growth and acts synergistically with chemotherapeutic drugs including taxol, irinotecan and gemcitabine [57]. OMP-18RS, is the only potential compound targeting the wnt pathway to make it to clinical phase I trials (NCT01345201) for the treatment of solid tumours and myeloid malignancies, suggesting potential use for HCC treatment.

Sorafenib has also been proposed as a potential wnt modulator, decreasing β-catenin and also expression of liver-specific wnt targets (GLUL, LGR5, and TBX3) in several HCC cell lines accompanied by reduced tumour volume *in vivo* using HepG2 xenografts in nude mice [58].

Several studies have also evaluated the significance of combination therapy for targeting the wnt pathway. A small molecular target, FH535 inhibits proliferation of HCC cell lines by inhibiting recruitment of β-catenin coactivators and also suppresses peroxisome proliferator-activated receptor (PPAR) signalling. Galuppo et al. [59] reported FH535 and sorafenib synergistically inhibited HCC cell line and liver cancer stem cells by targeting the RAS/RAF/MAPK and WNT/β-catenin pathways. Western blot demonstrated cleaved increased poly (ADP-ribose) polymerase (PARP) and reduced cyclin D1 and c-Myc.

Identification of pharmacological inhibitors of the wnt/βcatenin pathway is still underway. In the complex network of wnt ligands, receptors and β-catenin, preclinical studies have yielded promising results but wnt inhibitors targeting HCC have not yet reached clinical trials.

6.2. Immunotherapeutics

Immune checkpoints are emerging as promising targets for treatment of HCC. The immune system helps to distinguish body's own cells from foreign cells. To help it achieve this, immune checkpoints which are molecules on certain immune cells, need to be activated or inactivated to start an immune response. Cancer cells find ways to use such checkpoints to escape immune response.

Programed death-1 (PD-1) is a check-point receptor found on $CD8^+$T-cells and directs it from attacking other cells in the body. PD-1 binds to PD ligands (PDL)-1 and PDL-2. Cancer cells have high amount of PD-1 and PDL-1 which helps them evade immune response. In HCC, $CD8^+$ T-cells that express PD-1 is much higher in both tumour regions and peripheral blood compared to healthy controls [60, 61]. Additionally, several correlative studies have associated PD-1 and PDL-1 in tumours to be significantly associated with HCC recurrence, and poor prognosis [62, 63]. Monoclonal antibodies against PD-1 and PD-L1 have been developed and are under clinical trials in HCC patients.

Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) is another example of an immune check-point, which serves an inhibitory co-receptor that interferes with T cell activation and proliferation. CTLA-4 pathway downregulates an immune response by binding to CD80. Inhibiting the CTLA-4 pathway leads to T-cell activation and proliferation and may help generate memory T cells. Monoclonal antibody against CTLA-4 are also under trial in HCC [64].

Pembrolizumab and nivolumab are monoclonal antibodies targeting PD-1. A phase II study (KEYNOTE-224, NCT02702414) is currently underway to access the toxicity and activity of pembrolizumab in advanced HCC patients who have been treated with sorafenib. The primary objective of this study is to determine the objective response rate (ORR) of pembrolizumab given as monotherapy. Recently, a case study reported a decrease in tumour size and AFP levels in a 75 year old man with metastatic HCC who was treated pembrolizumab after failure to respond to sorafenib [65]. Another single-arm phase II trial of pembrolizumab is underway (Keytruda, NCT02658019) and is recruiting advanced HCC patients with unresectable HCC. The primary end points of this study are PFS, OS, RR, duration of response and toxicity.

A phase I/II study is underway (CheckMate040, NCT01658878) to evaluate the safety and tolerability of nivolumab. HCC patients who were either not responsive to sorafenib or failed sorafenib are also included in the study. Another phase III study (CheckMate-459, NCT02576509) is recruiting HCC patients to compare nivolumab with sorafenib as a first line treatment for advanced HCC patients.

Durvalumab is a monoclonal antibody targeting PDL-1. A phase II study (NCT02519348) is currently recruiting patients with unresectable HCC to evaluate durvalumab and tremelimumab either alone or in combination.

Tremelimumab is an inhibitor of CTLA-4. In a small pilot clinical trial including 21 patients with metastatic HCV-related HCC, tremelimumab induced a significant decrease in viral load

and showed promising partial response rate and disease control rate of 17.6% and 76.4%, respectively. The TTP was 6.5 months [66].

6.3. Epigenetic-based therapeutics

Chromatin remodelling is a critical epigenetic mechanism regulating gene expression and plays an important role in cell proliferation, differentiation and DNA repair. Epigenetics alters gene expression without any changes to the DNA sequence and involves the enzymatic covalent modification of histones such as methylation, phosphorylation and acetylation. Histone deacetylases (HDACs) remove the acetyl group from histones, making DNA more compact resulting in gene silencing. There are a total of 18 HDACs identified in mammals. Accumulating evidence suggests the overexpression of HDACs to be correlated advanced tumour stage, recurrence after surgery and poor prognosis in several cancers including HCC [67, 68]. For these reasons HDAC inhibitors may serve as potential therapeutic targets.

Currently 2 HDAC inhibitors have been approved by the FDA i.e. vorinostat and romidepsin, for treatment of cutaneous T cell lymphoma. These inhibitors demonstrate anti-tumour activity by means of histone hyperacetylation reducing DNA-histone affinity and allowing access to transcription factors enhancing gene expression. Currently, a phase I clinical trial (NCT01075113) is underway to evaluate vorinostat in combination with sorafenib in HCC. Romidepsin has not been tested in a clinical setting.

Aberrant up regulation of several HDACs (HDAC1, 2, 3, 4, 5, and 11) and changes in copy number of HDAC3 and HDAC5 have been reported in HCC. Treatment with panobinostat (HDAC inhibitor) demonstrated strong anti-tumour activity *in vitro* and *in vivo* and the effect was enhanced in combination with sorafenib [69]. In a recently completed phase I/II trial (SHELTER, NCT00943449), combination of resminostat (HDAC inhibitor) with sorafenib yielded a progression-free survival-rate of 12.5% for resminostat alone and 62.5% for resminostat plus sorafenib. Median TTP and OS were 1.8 months and 4.1 months for resminostat and 6.5 months and 8.0 months for the combination, respectively [70]. These results support further evaluation of HDAC inhibitors in clinical settings in HCC.

7. Conclusion

New therapeutic options are needed for the treatment of HCC despite the availability of sorafenib, which has limited survival benefits in advanced HCC patients. Several clinical trials are investigating the efficacy and tolerability of combining sorafenib with other agents. Future studies should continue to delineate dysregulated signalling pathways in hepatocarcinogenesis to introduce new molecular targets for therapeutic intervention. Simultaneously it is critical to identify biomarkers and/or aberrant genotypes that would predict clinical efficacy to these targeted agents. Much work also remains to evaluate the role of targeted therapy in adjuvant, neoadjuvant or metastatic settings to determine the most suitable combination of treatment. The battle against HCC is far from over and requires a multidisciplinary approach.

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Hepatocellular carcinoma (HCC) currently ranks as the third most common cause of death. As the primary malignancy of the liver is directly related to an underlying liver condition, its incidence and profile are expected to change soon. While effective prevention programs and antiviral therapies for hepatitis B and C will lower the incidence of HCC, emerging socioeconomic issues will deliver new at-risk populations.

Moreover, diagnostic techniques and protocols have undergone significant advancements. Reliance on contrast enhanced ultrasound has been re-evaluated, imaging methods being considered as sufficient diagnostic tools. Molecular characterization remains desirable, since chemotherapeutic agents still have limited applicability.

In light of recent diagnostic advancements and novel therapeutic solutions, it is our belief that a comprehensive update on recent paradigm shifts and interesting upcoming developments is highly needed.

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