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ΕΠΙΒΛΕΠΩΝ: Δρ. ΑΧΙΛΛΕΑΣ ΓΡΑΒΑΝΗΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

Η ΔΙΕΡΕΥΝΗΣΗ ΝΕΩΝ MICRORNA ΠΟΥ ΠΑΙΖΟΥΝ ΡΟΛΟ ΣΤΗΝ ΔΗΜΙΟΥΡΓΙΑ ΚΑΡΚΙΝΟΥ ΗΠΑΤΟΣ ΜΕΣΑ ΑΠΟ ΥΨΗΛΗΣ ΤΕΧΝΟΛΟΓΙΑΣ ΑΝΑΛΥΣΗ

ΑΛΕΞΑΝΔΡΑ ΔΡΑΚΑΚΗ

ΕΙΔΙΚΟΣ ΠΑΘΟΛΟΓΟΣ-ΑΙΜΑΤΟΛΟΓΟΣ-ΟΓΚΟΛΟΓΟΣ

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Η ΔΙΕΡΕΥΝΗΣΗ ΝΕΩΝ MicroRNA ΠΟΥ ΠΑΙΖΟΥΝ ΡΟΛΟ ΣΤΗΝ ΔΗΜΙΟΥΡΓΙΑ ΚΑΡΚΙΝΟΥ ΗΠΑΤΟΣ ΜΕΣΑ ΑΠΟ ΥΨΗΛΗΣ ΤΕΧΝΟΛΟΓΙΑΣ ΑΝΑΛΥΣΗ

ΠΕΡΙΛΗΨΗ

ΙΣΤΟΡΙΚΟ

Το ηπατοκυταρρικό καρκίνωμα ή ηπάτωμα είναι ο συχνότερος πρωτοπαθής κακοήθης όγκος του οργάνου αυτού (90-95%) και η συχνότερη αναλογικά θανατηφόρος κακοήθης νεοπλασία. Συγκεκριμένα ο καρκίνος του ήπατος είναι η δεύτερη αιτία θανάτου λόγω καρκίνου για τους άντρες και η έκτη αιτία για τις γυναίκες. Αυτές οι στατιστικές αναλύσεις αντικατοπτρίζουν την επιθετικότητα αυτού του όγκου καθώς και την εως τώρα έλλειψη αποτελεσματικών θεραπειών στο συγκεκριμένο πεδίο. Έχουν διεξαχθεί εκατοντάδες κλινικές δοκιμές είτε με συνδυασμό χημικοθεραπευτικών σχημάτων είτε με μικρομοριακούς αναστολείς και πρόσφατα αναστολείς του ανοσοποιητικού συστήματος. Παρ'όλα αυτά το μόνο φάρμακο εγκεκριμένο από τον ΕΟΦ για ανεγχείρητους ή μεταστατικούς όγκους είναι το sorafenib που είναι αναστολέας της τυροσινικής κινάσης.

Ο σκοπός μας ήταν να βρούμε γονίδια που παίζουν ρόλο στην δημιουργία του ηπατοκυτταρικού καρκίνου και έτσι κατευθύναμε τον στόχο μας στο να μελετήσουμε και να καταλάβουμε κάποια νέα μικρά γονίδια που δεν μεταφράζονται σε πρωτεΐνες και λέγονται microRNAs.

Τα microRNAs έχουν εμπλακεί στην παθογένεια διαφορετικών καρκίνων συμπεριλαμβανομένου αυτού του ήπατος. Συγκεκριμένες microRNA υπογραφές έχουν βρεθεί να είναι απορρυθμισμένες στους ασθενείς με ηπατοκυτταρικό καρκίνωμα και να συσχετίζονται με την επιβίωση.

ΜΕΘΟΔΟΙ

Σε αυτήν την μελέτη εξετάσαμε το ανθρώπινο microRNA γονιδίωμα. Επίπεδα έκφρασης των microRNA και γονιδίων μετρήθηκαν με ποσοτική πραγματικού χρόνου PCR σε ιστούς με ηπατοκυτταρικό καρκίνωμα και φυσιολογικούς ιστούς. Ο αλγόριθμος TargetScan χρησιμοποιήθηκε για να βρεθούν οι άμεσοι στόχοι καθοδικά του microRNA-9.

ΑΠΟΤΕΛΕΣΜΑΤΑ

Μέσα από υψηλής τεχνολογίας ανάλυση του ανθρώπινου microRNA γονιδιώματος βρήκαμε 28 microRNAs που είναι ρυθμιστές και προαγωγείς της επιθετικότητας των ηπατικών κυτταρικών σειρών. MiR-9, miR-21 και miR-224 ήταν οι 3 πρώτοι επαγωγείς της επιθετικότητας και η έκφραση τους ήταν πιο αυξημένη στους ιστούς με καρκίνο από ότι τους φυσιολογικούς. Ο συνδυασμός των κλινικών και μοριακών δεδομένων έδειξε ότι το mir-9 ήταν το πρώτο με κλινική και λειτουργική σπουδαιότητα. Τα επίπεδα του συσχετίζονταν με την σταδιοποίηση των ασθενών με ηπατοκυτταρικό καρκίνο. Είναι σημαντικό να σημειωθεί ότι η υπερέκφραση του miR-9 in vitro , επάγει στις SNU-449 και HepG2 κυτταρικές σειρές την κυτταρική ανάπτυξη, επιθετικότητα και ικανότητα τους να σχηματίζουν αποικίες σε μαλακό άγαρ. Μέσα από βιοπληροφορική και ανάλυση λουσιφεράσης βρήκαμε ότι η e-cadherin και το peroxisome proliferator-activated receptor alpha (PPARA) είναι οι άμεσοι στόχοι καθοδικά του microRNA-9. Αναστολή του PPARA καταστέλλει τα επίπεδα του mRNA της e-cadherin γεγονός που υποδηλώνει ότι το miR-9 ρυθμίζει την έκφραση της e-cadherin άμεσα μέσω σύνδεσης στο 3'UTR του γονιδίου και έμμεσα μέσω PPARA. Επιπρόσθετα η αναστολή της υπερέκφρασης του miR-9 μειώνει την ογκογονικότητα καθώς και την επιθετικότητα του όγκου. Τα επίπεδα έκφρασης του mRNA του PPARA και της e-cadherin ήταν μειωμένα στους ιστούς με ηπατοκυτταρικό καρκίνωμα σε σχέση με τους φυσιολογικούς και αντιστρόφως ανάλογα με τα επίπεδα του miR-9.

ΣΥΜΠΕΡΑΣΜΑ

Εν κατακλείδι, αυτή η μελέτη περιγράφει για πρώτη φορά τον σημαντικό ρόλο του μονοπατιού σηματοδότησης miR-9/PPARA/e-cadherin στην δημιουργία καρκίνου του ήπατος. Με βάση αυτήν την εργασία ο επιστημονικός στόχος μας είναι να χρησιμοποιήσουμε μη αναστρέψιμους αναστολείς του miR-9 σε κλινικές δοκιμές πρώτης φάσεως με ελπίδα να χρησιμοποιηθούν ως πιθανή θεραπεία του ηπατοκυταρρικού καρκίνου.

Identification of Novel MicroRNAs Involved in Hepatocellular Carcinogenesis by High Throughput Screening

ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death in men and the sixth in women. Those numbers reflect the aggressiveness of this disease while at the same time mirror the absence of effective therapeutic regimens. There have been conducted hundreds of clinical trials in which either combination chemotherapies or small molecule inhibitors and recently immune checkpoint inhibitors have been studied. However the only FDA approved drug for unresectable or metastatic HCC is sorafenib which is a tyrosine kinase inhibitor.

Our interest was to identify genes that play a role in liver oncogenesis and so we directed our focus to study a novel class of small non coding RNAs, the microRNAs.

MicroRNAs have been involved in the pathogenesis of different types of cancers, including liver cancer. Specific microRNA signatures have been identified to be deregulated in HCC patient tissues and also to correlate with different clinicopathological parameters including survival.

Having all this in mind our mission was to identify microRNAs that have both functional and clinical relevance in HCC and examine their downstream signaling effectors.

Methods:

In this study, we have screened the human microRNAome, aiming to identify microRNAs that are potent regulators of HCC invasiveness. Initially we received 24 tissues from patients with liver cancer along with 14 from adjacent non tumor specimens. RNA extraction was performed and microRNA and gene expression levels were measured by quantitative real-time RT-PCR. We determined the expression levels of miR-9, miR-21 and miR-224 in those samples. Simultaneously a novel microRNA library screen was done after plating SNU-449 liver cancer cells in 96-well plates. Those were transfected with a microRNA library consisting of 316 microRNA mimics and 2 negative controls. 48hrs post transfection we evaluated the SNU-449 cell invasiveness. We also performed invasion assays in SNU-449 cells 24hrs after transfection specifically with miR-9 and anti-miR-9 and their respective controls. A TargetScan algorithm was used to identify miR-9 downstream direct targets based on bioinformatics. Those analyses were validated by performing 3'UTR luciferase assay. Precisely, we transfected SNU-449 cells with the reporter vectors carrying the 3'UTR of CDH1 or PPARA. The constructs harbored the seed sequence of miR-9 (wildtype) or had a deletion of this sequence (miR-9 mutant). At 24 hours, they were transfected with miR-9 or miR-control and at 48 hours luciferase activity was measured. We have also performed transfection with microRNA mimic for mir-9 overexpression as well as microRNA inihibitor to suppress the miR-9 activity and observed the expression levels of the downstream targets CDH1, PPARA, PDK4 and vimentin. Furthermore we performed cell

proliferation as well as assessed sphere formation and colony assay in SNU-449 and HepG2 liver cancer cell lines, both of which were transfected with miR-9 and anti-miR-9 along with their respective controls.

Results: First we performed a high-throughput microRNA screen in SNU-449 liver cancer cells and assessed their invasiveness while secondly we evaluated in tissue the expression levels of the microRNAs derived from the above screen. Overexpression of miR-9 was found to be the top inducer of SNU-449 cell invasiveness, cell growth and their ability to form colonies in soft agar. Furthermore miR-9 levels were found in tissue to increase during HCC progression. Bioinformatics and 3'UTR luciferase analyses identified E-cadherin (CDH1) and peroxisome proliferator-activated receptor alpha (PPARA) as direct downstream effectors of miR-9 activity. Inhibition of PPARA suppressed CDH1 mRNA levels, suggesting that miR-9 regulates CDH1 expression directly through binding in its 3'UTR and indirectly through PPARA. On the other hand, miR-9 inhibition of overexpression suppressed HCC tumorigenicity and invasiveness. PPARA and CDH1 mRNA levels were decreased in HCC relative to controls and were inversely correlated with miR-9 levels.

Conclusions: Our study identified a novel microRNA signaling pathway, consisting of miR-9, PPARA and CDH1 that is deregulated in HCC patients affecting liver cancer cellular invasiveness and metastatic potential.

1. GENERAL SECTION

1.1 The normal liver.

1.1.1 The liver anatomy

The liver is the largest internal organ occupying the abdominal cavity and the largest gland in the body. It has a broad spectrum of functions whose role is vital for survival. The terminology related to the liver usually starts in *hepar-* or *hepat-* that originate from the greek word *hēpar* (ἧπαρ, root hepat-, ἡπατ-).

The liver lies below the diaphragm in the right upper quadrant of the abdomen. It normally weighs 1.44–1.66 kg (3.2–3.7 lb) occupying 2.5% of total body weight. It is a soft, pinkish-brown, triangular organ. It is divided by the falciform ligament into the right and left lobar segments. From the visceral surface there are two more lobes between the right and the left and those are known as the caudate lobe which is located superiorly and the quadrate lobe which is inferiorly. Based upon its blood supply or bile duct distribution the liver is subdivided into eight (Couinaud) lobes. Each of the lobes is made up of lobules. The lobules which are consistent of the hepatic cells are the functional units of the liver[1].

The liver lobes have two major types of cells which are the parenchymal cells, known as hepatocytes that occupy the 80% of the liver volume and the non-parenchymal cells some of which include the Kupffer cells and sinusoidal hepatic endothelial cells that line the liver sinusoid [2].

This organ has a unique dual blood supply from the hepatic arteries and the portal vein. The hepatic arteries carry the oxygenated blood coming from the aorta whereas the hepatic portal vein carries the blood coming from the gastrointestinal tract, pancreas and spleen, accounting for approximately the 75% of liver's blood supply. Important to mention is that its oxygen demand is equally met by both the liver arteries and the portal vein. The blood flows through the liver sinusoids to the central vein of each lobule. These consequently coalesce and form the hepatic veins that drain into the inferior vena cava.

The liver produces bile which is collected in bile canaliculi that eventually form bile ducts. Those ducts are called intrahepatic when are located within the liver and extrahepatic when are outside the liver. The intrahepatic ducts drain into the right and left hepatic ducts that coalesce and form the common hepatic duct. The common hepatic duct joins with the cystic duct, which drains the gallbladder and eventually form the common bile duct that drains into the second part of the duodenum at the ampulla of Vater.

1.1.2 The normal functions of the liver

The liver plays a pivotal role in various metabolic functions that include but are not limited to:

1. Maintenance of normal blood sugar levels through glycogenesis, glycogenolysis and gluconeogenesis

2. Synthesis, metabolism and degradation of proteins. Some characteristic examples include the production of albumin (which is the major osmolar component of the blood serum), insulin-like growth factor 1(which has an anabolic effect in adults and plays an important role during childhood growth), angiotensinogen (which is a hormone responsible for changes in blood pressure, regulated by renin) and thrombopoietin (which is a hormone that regulates the platelet production by the bone marrow)
3. Synthesis of lipoproteins, cholesterol and lipogenesis.
4. Production of coagulation factors including I (fibrinogen), II (prothrombin), V, VII, IX, X,XI, protein C, protein S and antithrombin.
5. Production and excretion of the bile that is necessary for digestion
6. Removal of bacteria, metabolic waste products, toxins, drugs as well as senescent red blood cells.
7. Conversion of ammonia to urea (urea cycle).
8. Glucuronidation of bilirubin facilitating its excretion into bile.
9. Storage of several vitamins (including vitamin A, D, B12, K), iron and copper.
10. Processing of nutrients that are absorbed from the digestive tract.
11. Immune system as it is a part of the reticuloendothelial system.

1.2 Hepatocellular carcinoma

1.2.1 Incidence and epidemiology

Hepatocellular carcinoma (HCC) is a primary liver cancer that originates in the hepatocytes. It is a fairly aggressive type of malignancy reflected by the fact that the incidence of this disease

approximates the number of deaths each year [3]. HCC is the sixth most common type of cancer worldwide with more than half a million new cases annually [4]. Based on the National Cancer Institute data, it is estimated that for the year of 2015 there are going to be 35,660 new cases with 24,550 deaths in the Unites States of America only.

Differently from other types of cancer, HCC has a unique geographic, ethnic, age and sex distribution [3]. It is postulated that the differences in the incidence of hepatitis B and Hepatitis C virus carriers worldwide contribute to the differences in liver cancer distribution. Therefore there are the high incidence regions such as in Asia (Korea and China) in which the death rates (which correlate with the incidence rates) are 23-150 cases per 100,000 people per year, the intermediate incidence regions such as South Africa and Austria with annual death rates ranging from 5-10 cases per 100,000 people and the low incidence countries such as the United States of America with 1.9 cases per 100,000 people per year [5]. The incidence rates are rising in both central Europe and United States of America likely due to the growing population with longstanding chronic hepatitis C, due to the large influx of immigrants from areas with large prevalence of hepatitis B infection such as the East Asia, as well as due to an increase in nonalcoholic fatty liver disease [6]. HCC is the fifth most common type of cancer in males and the seventh most common in females. Regardless of the regional incidence, there is male predominance with a male to female ratio of approximately 4:1. It is believed that high estrogen levels along with low testosterone levels have a protective role leading to a lower risk for developing HCC in the female population[7].

Furthermore regardless age and sex, the Asians have a rate of HCC two times higher than African Americans who similarly have two time higher rates than whites. As aforementioned ethnic factors play an important role as incidence rates could vary according to ethnic origins

even in the same population. Some characteristic examples are that Ethnic Japanese in Japan have higher incidence of HCC compared to those leaving in Hawaii who in turn have higher incidence than those leaving in California. Similarly Jews of Africa or Jews of Asia leaving in Israel have higher incidence compared to those of European descent[8] **(Table 1).**

Table 1. Incidence of hepatocellular carcinoma by sex in various countries and ethnic groups (100,000 /year)

COUNTRIES	MALES	FEMALES
Mozambique	112.9	30.8
Zimbabwe	64.6	25.4
Argentina	9.9	5.8
United States		
Chinese	19.1	3.6
Black	3.9	1.8
Japanese	3	0.4
Caucasian	2.9	1.1
Switzerland	10.2	1.5
Germany	4.5	1.7
United Kingdom	1.6	0.8
China	34.4	11.6
Japan		
Miyagi	11.2	4
Nagasaki	25.8	7.9
India	4.9	2.5

1.2.2 Risk Factors

There are several risk factors that have been identified to date and are considered responsible for increasing the predisposition of an individual person to develop hepatocellular carcinoma. By far the most common cause of HCC is chronic infection with the hepatitis B and C viruses. In up to 80% of the cases of liver cancer there is underlying cirrhosis. In the background of cirrhosis, nodules evolve to low-grade dysplastic nodules, then to high grade dysplastic nodules and finally to cancer[9]. The rest 20% of patients who do not have cirrhosis there is not any clear

etiology of their HCC development neither of natural history of the disease. It is estimated that at least up to 30% of liver cancers in the United States of America are due to HCV infection [10]. As with any other type of cancer there are several somatic mutations in HCC with the most frequently mutated genes being p53, PIK3CA and beta-catenin. Other genes that likely play a role in liver cancer pathogenesis are c-myc, cyclin D1, Rb1, p16, PTEN, SOCS, e-cadherin, IGFR-II/M6PR,EGFR and TGF-beta[9]. Therefore all these genes and genetic pathways which are involved are likely being deregulated by the various etiologic factors that have been identified to date and cause HCC **(Table 2).** Here we will discuss more extensively some of those.

Table 2. Conditions associated with hepatocellular carcinoma

CONDITIONS	RISK
CIRRHOSIS	
HEPATITIS B VIRUS	HIGH
HEPATITIS C VIRUS	HIGH
ALCOHOL	HIGH
AUTOIMMUNE CHRONIC ACTIVE HEPATITIS	HIGH
CRYPTOGENIC CIRRHOSIS	MODERATE
CIRHOSSIS DUE TO NASH	MODERATE
PRIMARY BILIARY CIRRHOSIS	LOW
METABOLIC DISORDERS	
HEREDITARY HEMOCHROMATOSIS	HIGH
HEREDITARY TYROSINEMIA	HIGH
a1-ANTITRYPSIN DEFICIENCY	MODERATE
ATAXIA TELANGIECTASIA	MODERATE
TYPES 1 AND 3 GLYCOGEN STORAGE DISEASE	MODERATE
GALACTOSEMIA	MODERATE
CITRULLINEMIA	MODERATE
HEREDITARY HEMORRHAGIC TELANGIECTASIA	MODERATE
PORPHYRIA CUTANEA TARDA	MODERATE
WILSON'S DISEASE	LOW
OROTIC ACIDURIA	MODERATE
ALAGILLE'S SYNDROME (CONGENITAL CHOLESTASIS)	MODERATE
ENVIRONMENTAL	
THOROTRAST	MODERATE

ANDROGENIC STEROIDS	MODERATE
CIGARETTE SMOKING	LOW TO MODERATE
AFLATOXIN	MODERATE
RYRROLIZIDINE ALKALOIDS	UNKNOWN
CYCASIN	UNKNOWN
N-NITROSYLATED COMPOUNDS	UNKNOWN

1. Hepatitis B Virus Infection

Several studies including case-control and cohort studies suggest a strong association between chronic hepatitis B carrier rates and increased risk of HCC development. In HBV-associated HCC half of the patients have chronic active hepatitis compared to those with HCV-associated HCC who mainly have frank cirrhosis. It is believed that the genome of HBV is integrated into host DNA in a random fashion, causing microdeletions that can target cancer-related genes and transforming the normal hepatocytes into malignant cells. The related genes include MAPK1, PDGFRb, TERT and others. Likely the malignancy arise from grounds of hepatic destruction with subsequent replicative repair and proliferation that lead to the accumulation of mutations associated with cancer development[11]. Kim et all have described a potential mechanism of HBV induced HCC based on which the hepatitis B viral protein x (HBx) directly binds and inactivates p53, causes transcriptional activation and alteration of the expression of growth control genes such as SRC tyrosine kinases, Ras, Raf, MAPK, ERK, JNK and others [12]. It is estimated that the average age for HBV associated HCC is around 52 years.

Liver cancer incidence is directly related to the serum levels of HBV DNA however the role of HBV as a direct carcinogen is unclear[13]. Taiwanese male who are carriers for the hepatitis B surface antigen (HBsAg)-positive have a 98-fold greater risk for HCC compared to HBsAg-negative individuals[14]. Dodd et al showed that there was a minimum relative risk of 12.7 for HCC when they evaluated at American Red Cross Centers asymptomatic HBsAg-positive blood donors compared with HBsAg-negative individuals[15]. HBsAg-positive patients have even higher

risk if they are males, above 45 years of age, with underlying cirrhosis and positive family history of the disease[16]. The risk of developing HCC is lower in inactive carriers compared to those with active hepatitis but still higher in comparison to the general population[17]. Interestingly the World Health Organization (WHO) is the sponsor of an ongoing large scale intervention in Asia involving HBV vaccination of the newborn that will eventually lead to a decrease in the incidence of HCC in the vaccinated population. Finally in the setting of hepatitis B infection even a stronger risk factor is the co-infection with hepatitis C, concurrent continuous alcohol consumption or underlying cirrhosis from any cause[18].

2. Hepatitis C Virus Infection

HCV is an RNA virus and therefore cannot integrate into the hepatocyte DNA but can interact with the endoplasmic reticulum of the host cell, causing stress and leading to procarcinogenic changes. Furthermore the HCV core proteins interact with the MAPK signaling pathway and so modulate the cell proliferation in a direct way. Also the NS5A protein inactivates p53 and finally the E1/E2 HCV proteins inhibit apoptosis[11].

Given the fact that patients with HCV are in a state of chronic infection it is postulated that their immune system is also chronically activated that likely also plays a significant role in tumor progression. The hepatitis C virus itself causes rapid cellular turnover and a chronic inflammatory state which ultimately causes fibrosis and eventually cirrhosis[19]. Several lines of evidence have shown that almost every patient with either advanced liver fibrosis or cirrhosis will eventually develop liver cancer[20]. Interestingly the degree of liver inflammation correlates with the prognosis once cancer is diagnosed[21]. Similarly to HBV infection, the chronic tissue

destruction with constant regeneration has as a consequence the accumulation of mutations leading to hepatocarcinogenesis.

Hepatitis C virus is known to cause chronic infection up to 60-80% of the cases and compared to HBV infected patients, those with HCV have a 20-fold increase risk of developing cirrhosis which usually presents with a more advanced stage[22]. The average age for HCV associated HCC is approximately 62 years which is a later pattern of presentation compared to HBV. At this point it is also important to point out that the estimated interval between HCV associated transfusion which is the time of contraction of this virus and eventually the development of HCC is approximately 3 decades. Therefore compared to 40- 50 years for HBV, the HCV-based HCC evolves much faster.

3. Tobacco and Alcohol Abuse

Several studies described a strong correlation between cigarette smoking and increased risk for development of liver cancer[23] . In the same context of toxin abuse, long standing alcohol consumption is known to cause oxidative stress in the normal hepatocytes that consequently promotes inflammation, fibrosis and eventually cirrhosis. Alcohol induced cirrhosis creates a permissive HCC microenvironment. The mechanism based on which this happens is the following: The cytochrome P-450 and the enzymes alcohol dehydrogenases metabolize the ethanol leading to production of reactive oxygen species and acetaldehyde which in turn binds to DNA and proteins of the cell, damages mitochondria and leads to apoptosis. On the other hand the reactive oxygen species lead to protein oxidation, lipid peroxidation and DNA adducts[24]. The oxidative stress leads to decreased STAT1-directed activation of IFN gamma signaling which plays a role in the damage of the hepatocytes, the promotion of the development of fibrosis and

finally cirrhosis[25]. Furthermore the monocytes are being activated by alcohol leading to inflammatory cytokine release. As a consequence the Kupffer cells are being activated causing a further release of cytokines and chemokines and eventually liver cell necrosis.

4. Non-alcoholic fatty liver disease (NAFLD) (also known as Non-alcoholic steatohepatitis (NASH)

One of the most common conditions in the Western world nowadays is the metabolic syndrome that is defined by the presence of obesity, insulin resistance, hypertriglyceridemia and low levels of high-density lipoprotein (HDL). The hepatic manifestation of this syndrome is Non Alcoholic Fatty Liver Disease[26]. In this condition the increased free fatty acids in the liver activate inflammatory pathways via NF-kB activation. This inflammatory process leads to cirrhosis and eventually cancer. NAFLD related HCC is linked mainly to male predominance, hypertension, diabetes and obesity which could be expected given the causal-effect relation of those factors with the metabolic syndrome[27]. Therefore metabolic syndrome is indirectly a risk factor for HCC with an odds ratio of 2.1 [28].

4. Chronic (non-viral) Hepatitis and Cirrhosis

Patients with chronic liver disease of any cause have an increased risk of developing liver cancer. It is worthwhile it to mention that those patients with elevated serum levels of alpha-fetoprotein (AFP) have a higher risk of developing liver cancer compared to those with low or normal serum AFP concentrations (< 20mcg/L)[29]. As it has been aforementioned up to 80% of cases of HCC are due to cirrhosis. In Southeast Asia patients with HCC most commonly have macronodular cirrhosis while in Europe and the United States usually have micronodular cirrhosis especially

due to alcohol consumption[30]. There is an extensive list of conditions that have been associated with HCC including autoimmune chronic active hepatitis, metabolic diseases which are less common, cryptogenic cirrhosis in which the underlying cause has not been identified and environmental factors. Among all the causes of chronic hepatitis those that are linked with the highest risk of developing liver cancer are hereditary hemochromatosis, hepatitis C infection and cirrhosis, age above 55 years and alcohol abuse [31].

5. Chemical Carcinogens

Aflatoxin B1 is the product of the Aspergillus fungus and it is thought to be the most potent natural chemical carcinogen. The aflatoxin along with the mold can be found in hot and humid areas and in a variety of stored grains such as rice in unrefrigerated conditions. Therefore in several areas of Africa and China there is aflatoxin contamination of the foodstuffs with a good correlation with incidence rates of HCC. Interestingly, in hyper endemic areas even ducks and other farm animals do develop HCC. Other potent carcinogens are natural products of plants, fungi and bacteria such as safrole, tannic acid and pyrrolizidine alkaloids.

There is a large amount of evidence that anabolic steroids are considered carcinogens and linked to liver cancer while estrogens are known to cause benign adenomas [32]. Finally in the western world and developed countries environmental pollutants such as pesticides and insecticides are considered hepatic carcinogens.

1.2.3 The Differential Diagnosis of liver lesions and the histopathology of Liver Cancer

There is a great range of abnormalities that could present as a solitary liver lesion that are non-neoplastic, including inflammatory pseudo-masses, hepatic cysts, focal nodular hyperplasia and pseudo tumors associated with inflammation or infection, however those are more rare and the possibility of malignancy needs to be thoroughly investigated.

The tumors of the liver, like in every other tissue or organ, are classified based on the cells of origin and are categorized either as benign or malignant. Approximately 85-95% of liver tumors are malignant epithelial neoplasms with an 1-3% malignant mesenchymal neoplasms and a 6-12% being benign tumors of epithelial origin **(Table 3).**

Typically primary liver tumors are often centrally located and could be solitary lesions and exophytic while tumors metastatic to the liver tend to be more peripheral, are multiple and cause umbilication of the liver surface.

The liver is one of the most common metastatic sites. The types of cancer with increased propensity to spread to the liver or adjacent biliary tree in decreasing order of frequency are lung cancer, colon cancer, pancreatic cancer, breast cancer and gastric cancer. Typically metastases tend to occur from the various primary tumors by hematogenous spread through the portal vein to the liver [33]. On the other hand HCC tends to spread via the lympatics to the lymph nodes around the liver followed by the peritoneal cavity and the lungs. A fairly characteristic feature of

HCC, that is a negative prognostic factor for resection or liver transplantation, is the invasion mainly of the portal vein as well as the hepatic vein.

Table 3. Hepatic Neoplasms

BENIGN TUMORS	
HEPATOCELLULAR HYPERPLASIA:	Microgenerative nodule
	Nodular hyperplasia
	Mixed hamartoma
HEPATOCELLULAR ADENOMA	Typical: associated with anabolic steroids
HEPATIC CYST	Simple, Polycystic
BILE DUCT ADENOMA	
BENIGN MESENCHYMAL TUMORS	Mesenchymal hamartoma
	Hemangioma
	Infantile Hemangioendothelioma
	Lymphangiomatosis
	Lipoma
	Leiomyoma
	Fibroma
	Myxoma
	Inflammatory pseudotumor
PRIMARY MALIGNANT EPITHELIAL TUMORS	
HEPATOCELLULAR CARCINOMA VARIANTS	fibrolamellar
	spindle cell
	giant cell
	clear cell
	carcinosarcoma
	sclerosing hepatoblastoma
CHOLANGIOCARCINOMA	
SQUAMOUS CELL CARCINOMA	
HEPATIC CYSTADENOCARCINOMA	
PRIMARY MALIGNANT MESENCHYMAL TUMORS	
Angiosarcoma	
Leiomyosarcoma	
Hemangioendothelioma	
Malignant schwannoma	

Fibrosarcoma
Malignant fibrous
Histiocytoma
Lymphoma
Osteosarcoma
Rhabdomyosarcoma
Mesenchymal sarcoma

Regarding the pathology of the hepatocellular carcinoma it is important to mention initially that it can be found either as a single lesion or clusters of multiple nodules. Those clusters are due to metastatic spread or as a result of multicentricity of the tumor. There is a great variety of histologic types ranging from undifferentiated to well, to moderate, to poorly differentiated. In more details, in the differentiated type the hepatocytes tend to grow characteristically in sheets that are separated by inconspicuous sinusoids. In the undifferentiated or poorly differentiated type the liver cancer cells are pleomorphic, vary in shape, size, morphology and occasionally there may be present some giant cells. Moderately differentiated HCC could present as a mixture and appear solid, scirrhous or clear celled. Finally there is a variation of HCC known as fibromellar variant of hepatocellular carcinoma that usually occurs in earlier age than the typical HCC. The histopathologic characteristics of this type is the presence of eosinophilic tumor cells in a lamellar pattern. In order to distinguish primary HCC from metastatic lesions specialized immunohistochemical staining has been used. In more details, presence of alpha-feroprotein (AFP) and polyclonal carcinoembryonic antigen (CEA), loss of reticulin staining and flow-cytometric DNA analysis are useful for diagnosing primary tumors [34,35]. Finally the proliferation rate of the cancer cells have also a prognostic implication and can be detected by the cell cycle stains Ki67 and PCNA. Patients with low DNA synthetic capacity have a lower risk of developing intrahepatic spread and the 2-year survival post-surgery is greater than those individuals with rapid cell proliferation.

1.2.4 Clinical Features

1.2.4.1 Common Symptom and Signs

Like many other type of cancer, liver cancer could have a non specific presentation with vague symptoms and mainly related to the catabolic state due to the presence of malignant cells. Those nonspecific and non-pathognomonic symptoms are loss of appetite, weakness, generalized fatigue, weight loss, muscle wasting and cachexia that could be present in other tumors. However in any case and specifically in a patient with underlying cirrhosis or chronic liver disease those symptoms should alert both the patient and the caring physician for further investigation. On the other hand symptoms specifically due to HCC are abdominal pain which in fact is the most common presentation as well as a sensation of abdominal swelling and fullness, palpable masses mainly in the right upper quadrant, jaundice, nausea, vomiting, hematemesis and melena [36].

In more details, the abdominal swelling could be due to an underlying ascites likely as a consequence either of chronic liver disease or due to rapid tumor expansion. A more dramatic presentation that could be potentially fatal is central necrosis or acute hemorrhage into the peritoneal cavity. It is important to keep in mind that patients with underlying end stage liver disease are also thrombocytopenic, coagulopathic due to severe deficiency of clotting factors and vitamin K and therefore have increased tendency to bleed either as a consequence of a diagnostic liver biopsy or spontaneously in the tumor bed of a highly vascular rapidly expanding tumor leading to hemoperitoneum. Jaundice is not as frequent with only 10% of patients complain of

this. The mechanism by which jaundice occur is through obstruction of the main intrahepatic ducts, of the common hepatic duct at the porta hepatis, infiltration into the biliary radicals or rarely due to blood into the biliary tree. Hematemesis can occur in the setting of an underlying cirrhosis and portal hypertension that eventually lead to the development of esophageal varices. Up to 20% of patients have bone metastasis in autopsies however only 3-12% of those complain of bone pain. Rarely patients could present with respiratory symptoms such as dyspnea or pleuritic pain which are due to elevated hemidiaphragm from hepatomegaly or rib metastasis respectively.

One of the most common signs at the time of diagnosis of liver cancer in 50-90% of cases is hepatomegaly that could be fairly prominent especially in endemic areas of HCC or in more neglected cases. Up to 60% of patients have ascites which is as expected due to the underlying chronic liver disease and could even precede the diagnosis of cancer or could be a more dramatic presentation due to hemoperitoneum. Splenomegaly is the result of portal hypertension in patients who already had end stage liver disease and cirrhosis and as expected those patients would also have cytopenias due to splenic sequestration. On the other hand acute splenomegaly could be due to portal vein occlusion by the regional lymph nodes or the rapidly growing tumor and in such cases will be associated with acute pain. A 10-50% of cases may present with fever. The explanation of the mechanism of fever is not well described but it has been postulated that tumor necrosis and cytokine release is a potential cause. In 6-25% of cases, an experienced physician could identify abdominal bruit which is due to increased tumor vascularity. As aforementioned hepatocellular carcinoma tends to invade the portal vein and hepatic veins. In such cases occlusion or thrombosis of the hepatic veins that presents classically with a large tender liver and tense ascites is known as the Budd-Chiari syndrome. A fairly rare sign is the

presence of supraclavicular lymph nodes known as Virchow-Trosier nodes. In the literature cutaneous metastasis have been described as skin nodules with a reddish-bluish hue. Since the vast majority of patients with HCC have underlying liver disease for one reason or another, as expected those patients will also have the signs related to end organ damage that include palmar erythema, asterixis, jaundice, gynecomastia, dilated abdominal veins, testicular atrophy and peripheral edema.

1.2.4.2. Paraneoplastic symptoms

What is really fascinating in cancer presentation in several types of malignancies including hepatocellular carcinoma are the paraneoplastic symptoms. There is a great variety of biochemical abnormalities that could be a paraneoplastic manifestation of HCC and those include hypoglycemia, hypercalcemia, erythrocytosis, hypercholesterolemia in up to 40%, dysfibrinogenemia, carcinoid syndrome, increased thyroxin-binding globulin, porphyria cutanea tarda and sexual changes including gynecomastia, testicular atrophy and precocious puberty.

In more details, the hypoglycemia could be mild in cases of rapidly growing tumors while in slowly growing HCC it could be severe hypoglycemia as a result of defective processing of precursor to insulin growth factor-II (pro-IGFII). Hypercalcemia could be due to parathyroid hormone related protein (PTH-rp) production, of course in the absence of osteolytic lesions, in which case there will be a dual explanation of the high calcium levels. Erythrocytosis or polycythemia occurs in up to 10% due to the production by the tumor of erythropoietin-like substances. Finally black Africans could present with dermatopathic lesions such as pityriasis

rotunda, which is a rash with single or multiple oval or round scaly and hyperpigmented lesions on the trunk or thighs.

1.2.5 Staging Systems

Several staging systems for hepatocellular carcinoma have been described with the most commonly used being the American Joint Committee on Cancer/tumor-node-metastasis (AJCC/TNM), the new Cancer of the Liver Italian Program system (CLIP), the Barcelona Clinic system (BCLC) and the Okuda Staging system [36,37] . None of those systems have been universally adopted to predict the prognosis but there are certain features that are considered important determinants of survival. Those include the severity of the underlying liver disease, the size of the tumor, extension of the tumor to adjacent organs and the presence of metastasis. Finally, the ALBI (Albumin-Bilirubin) grade is a new evidence based score that needs to be validated but will eventually allow a more objective assessment of the severity of liver dysfunction in patients with HCC that have received various treatments.

Due to the non-specific presentation of HCC in most cases the diagnosis is being made late in the course of the disease when the tumor is usually advanced and therefore the median survival following diagnosis ranges from 6 to 20 months [37].

Based on the AJCC/TNM staging system tumors with a better prognosis are those with stage I and single lesions of less than 2cm in size without vascular invasion. On the other hand poor prognostic features are multiple tumors, presence of vascular invasion and lymph node involvement. Patients with stage III disease and lymph node involvement have a really poor

prognosis with a fairly small percentage surviving more than 1 year while less than 10% of those with stage IV have 1 year survival. Since vascular invasion and lymph node involvement is usually a pathologic and not necessarily always a radiographic diagnosis, full staging could be considered accurate only after surgical excision of the tumor. Of course this approach is clinically important for early stage tumors since for locally advanced or metastatic disease the surgery has no role in the treatment algorithm as the approach is usually palliation. Based on the TNM system the 5 year survival is 50-70% for stage I, 30-50% for stage II, 20-30% for stage IIIA, 10-20% for stage IIIB, 5-10% for stage IIIC and less than 2% for stage IV disease. The AJCC staging system has been validated and to date is considered the most accurate mainly for those patients who undergo either hepatic resection or liver transplantation[38] **(Table 4).**

Table 4. American Joint Commission on Cancer Staging (AJCC)/TNM system

PRIMARY TUMOR (T)			**STAGE GROUPING**			
Tx	Primary tumor can not be assessed					
T0	No evidence of primary tumor		I	T1	N0	M0
T1	Solitary tumor without vascular invasion		II	T2	N0	M0
T2	Solitary tumor with vascular invasion		IIIA	T3	N0	M0
	or multuple tumors less than 5cm		IIIB	T4	N0	M0
T3	Multiple tumors more than 5cm or		IIIC	Any T	N1	M0
	tumor involving a major branch of		IV	Any T	Any N	M1
	the portal or hepatic veins					
T4	Tumors with direct invasion of adjacent organs					
	or with perforation of visceral peritoneum					
REGIONAL LYMPH NODE (N)						
Nx	Regional lymph nodes cannot be assessed					
N0	No regional lymph node metastasis					
N1	Regional lymph node metastasis					
DISTANT METASTASIS (M)						
Mx	Distant metastasis cannot be assessed					
M0	No distant metastasis					
M1	Distant metastasis					

At this point is important to point out that for patients with underlying liver disease, the liver function and presence or absence of cirrhosis is what determines the prognosis of liver cancer and the life expectancy and not necessarily the staging based on the TNM system[39]. In such cases the Okuda and CLIP system are more accurate.

Okuda et al described the Okuda staging system in which the patients are not stratified based on the vascular invasion or the presence of lymph node involvement like the TNM staging system. Okuda system takes under consideration apart from the tumor size also some important clinical characteristics which are mainly related to the underlying liver disease and include serum albumin and bilirubin levels as well as the presence of ascites. Therefore this is a clinical scoring system as patients staged according to this system are not surgical candidates **(Table 5).**

Table 5. The Okuda Staging System

Parameter	Value	Points
Tumor Size	>50%	1
	<50%	0
Ascites	Present	1
	Absent	0
Serum Albumin	>3	0
	<3	1
Serum Bilirubin	>3	1
	<3	0
Stage	**Points**	
1	0	
2	1 to 2	
3	3 to 4	

Based on the Okuda system untreated patients have approximately a median survival of 8, 2 and less than 1 month for stages I, II and III respectively. Patients with Okuda stage III disease which

is the most advanced have by default poor prognosis since on one hand they cannot undergo resection with a curative intent and on the other hand due to liver dysfunction chemotherapy is contraindicated[40].

The CLIP score is most recently developed and is being used to predict survival especially for patients who are not surgical candidates. Studies have showed that it is better compared to TNM and Okuda systems. It combines the severity of cirrhosis with some tumor related features including AFP levels, tumor morphology and portal vein thrombosis **(Table 6).** The CLIP has a prognostic score ranging from 0-6 and that total score is a result of adding the subscores. In one study, median survival was 36, 22, 9, 7, and 3 months for patients in CLIP categories 0, 1, 2, 3, and 4 to 6, respectively.

Table 6. Cancer of Liver Italian Program (CLIP) Staging System

		POINTS	POINTS
VARIABLES	0	1	2
1. Morphology and hepatic replacement	Single < 50%	Multiple <50%	>50%
2. Child Pugh Score	A	B	C
3. AFP (ng/mL)	<400	>400	

Finally, based on the American HepatoPancreatoBiliary Association the recommendation is to use different staging systems for different patients. The AJCC TNM staging system predicts prognosis better than do the clinical systems, especially in post transplant patients or after hepatic resection. On the other hand non surgical candidates and patients that will undergo either localized therapy (TACE, RFA) or chemotherapy should be stratified based on the Okuda, Barcelona, and CLIP system[41].

1.2.6 Diagnosis

Prior to initiating any treatment and making a definite decision about the management of a patient with any malignancy it is critically important to have a definitive diagnosis. The gold standard approach is to confirm with biopsy by obtaining the involved tissue. However biopsy could be deferred in special cases. The American Association for the Study of Liver Disease (AASLD) Practice Guideline on Management of HCC outlines more criteria for the diagnosis of HCC which include a combination of the tumor size, a serologic assay and radiographic findings that will be discussed in more details later. Therefore based on the AASLD guidelines in patients with underlying cirrhosis, a 2 cm hepatic mass with characteristic radiologic appearance and serum AFP level of more than 200ng/mL the diagnosis is almost definite and therefore biopsy is not essential. For liver lesions between 1 and 2 cm image guided biopsy is recommended but as it will be discussed later even noninvasive radiographic modalities could lead to the diagnosis. Finally for lesions of less than 1cm the likelihood of HCC is low and so in such cases the recommendation is to follow up closely every six months unless there is a change in the clinical presentation.

Since biopsy is the gold standard approach for definite diagnosis, it is important to mention that a biopsy as a procedure itself is fairly challenging for several reasons in this patient population. First of all liver tumors are hypervascular and therefore there is a higher tendency to bleed

especially if we take under consideration the fact that those patients have chronic liver disease that leads to thrombocytopenia and deficiency of liver-dependent clotting factors. On top of that a more practical issue is that given the location of the liver and therefore of the tumor it is not possible to apply pressure post procedure to control any local sign of bleeding making the risk of hemoperitoneum possible.

Recently several criteria and guidelines have been revised based on which non-invasive diagnostic modalities are being used in the algorithm of diagnosing hepatocellular carcinoma in patients with chronic liver disease, including cirrhosis. The diagnostic imaging criteria of HCC rely on the characteristic appearance of HCC on the dynamic multiphase contrast-enhanced CT scans or MR images[42]. The qualitative criteria take into consideration the relatively decreased presence of contrast agent in most HCC compared with surrounding liver tissue during portal vein and equilibrium phase imaging along with their common differential increased arterializations[43,44]. The aforementioned qualitative criteria are fairly important since a rapidly growing nodule that is at least 1 cm and exhibits those should be considered as HCC. It is required that all the nodules regardless of the size to have late arterial phase enhancement; however there is a small percentage of tumors that appear isointense or even hypo intense in arterial phase and later phase imaging[45].

1.2.7 Current Therapies

For the treatment of hepatocellular carcinoma a multidisciplinary team is absolutely necessary since the challenges that the treating physicians have to face are multiple. The reason for that is that the disease itself is fairly aggressive so the patient needs to be managed in a timely manner

but in the same setting most of the cases have an underlying liver disease. The stage of the tumor along with the liver function are the major determinants of which treatment modality could be used and which is contraindicated. An overview of the different treatment options are being described in table and a detailed description will follow [46] **(Table 7).** Early stage tumors can be managed with several approaches including surgical excision, radiofrequency ablation and local injection and liver transplantation[47]. What is critically important to keep in mind is that the ideal approach is to choose the treatment that allows for maximal sparing of the liver parenchyma and at the same time to have local control of the disease that will give the option to the patient to undergo liver transplantation if he or she meets the appropriate inclusion criteria.

Table 7. Treatment Options for Hepatocellular Carcinoma

SURGERY
Partial Hepatectomy
Liver Transplantation
LOCAL ABLATIVE THERAPIES
Cryosurgery
Microwave ablation
Ethanol Injection
Acetic Acid injection
Radiofrequency ablation
REGIONAL THERAPIES: Hepatic artery transcatheter treatments
Transarterial chemotherapy
Transarterial embolization
Transarterial chemoembolization
Transarterial radiotherapy
Y90 microspheres
I131 lipiodol
CONFORMAL EXTERNAL-BEAM RADIATION THERAPY
SYSTEMIC THERAPIES

Chemotherapy
Immunotherapy
Hormonal therapy+growth control
SUPPORTIVE CARE

1.2.7.1 Surgery

Surgical excision is one of the main approaches for treating an early stage tumor. The primary goal is to obtain 1-cm margin of normal tissue surrounding the tumor. As expected this is not always possible and is dependent on the location of the lesion. Centrally located tumors may require a lobectomy while large tumors could be managed with extended hepatectomy. The mortality rate in a major hepatectomy is between 5-10%, mainly, due to the risk of fulminate liver failure in the setting of an underlying liver disease. Other potential complications are respiratory failure (including pneumonia, acute respiratory distress syndrome), ascites, infection or cardiovascular complications [48]. One of the major prognostic factors for tolerance of such surgeries is the Child-Pugh classification of liver failure **(Table 8).** Therefore patients with Child-Pugh A who do not have portal hypertension are considered appropriate candidates while those with portal hypertension may not tolerate the surgery well. Patients with Child-Pugh B and C, those with ascites and history of variceal bleeding should be given the alternative option of liver transplantation if indicated.

Table 8. Child-Pugh Classification of Cirrhosis

Measurement	1 point	2 points	3 points
Bilirubin (mg/dL)	1-1.9	2-2.9	>2.9
PT prolongation	1 to 3	4 to 6	>6
Albumin (g/dL)	>3.5	2.8-3.4	<2.8
Ascites	None	mild	mod/severe
Encephalopathy	None	Grade 1 or 2	Grade 3 or 4

GRADE A	5-6 points
GRADE B	7-9 points
GRADE C	10-15 points

A safer maneuver that can be done preoperatively that will allow for a safer resection is occlusion of the portal vein. That will lead to atrophy of the HCC-involved lobe and compensatory hypertrophy of the uninvolved liver parenchyma[49].

1.2.7.2 Liver Transplantation

The National Institute of Health Consensus Conference on liver transplantation in 1983 concluded that one of the most effective treatment options for patients with early stage HCC (stage I, II) that cannot be surgically resected is liver transplantation. That approach became standard of care since then for suitable patients even if the risk of recurrence was inevitable for a great percentage of cases. Several studies took place since then that showed that for patients with a single lesion of 5cm or more, or multifocal disease limited to more than three lesions, each 3 cm or more resulted in a 5 year disease free survival of more than 70% [50]. Therefore as of 2001 the indications for liver transplantation in patients with HCC include: the tumor should be up to 5cm, the patient is not a liver resection candidate, there is no macrovascular involvement neither involvement of locoregional lymph nodes and organs including lungs, bones or other abdominal organs. Based on those restrictions the aforementioned statistical data are being replicated and the 5 year tumor-free survival ranges between 70-75% [51]. At this point is very important to mention that patients who are otherwise candidates for liver transplantation are waiting too long and inevitably progress during that period. Therefore the caring physicians should be alerted and upfront should use a variety of non-resection therapies that will keep the tumor under local control and the patient

suitable candidate to remain in the waiting list. The local therapies that are being used as a "bridge" to transplant are radiofrequency ablation, ethanol injection and transarterial chemoembolization that will be discussed below.

1.2.7.3. Local ablative therapies

There are several local ablative therapies with the one of the most commonly used the radiofrequency ablation (RFA). The principle of this technique uses heat that thermally ablates the tumors [52]. Specifically a thin probe is inserted into the tumor. Needle electrodes are deployed to adjustable distances and deliver an alternating electrical current between 400 to 500 Hz. The current induces agitation of the particles of the surrounding tissues generating frictional heat that leads to necrosis of the tumor. The size of the necrosis depends on the length of the deployment of the electrodes and to date the maximum area of necrosis that could be achieved is 7 cm, which is suitable for a tumor up to 5cm. However, ideally should be used for small tumors up to 3cm that are located deeply in the liver parenchyma and away from the hepatic hilum. The downside of the technique is that when it takes place close to large vessels, those could act as heat sinks. In such cases there is not adequate cytoreduction of the cells adjacent to these structures [53]. Other potential adverse effects from the procedure is bile duct injury and obstruction when the tumors are located close to the main portal pedicles. A theoretical risk is needle track tumor seeding however that risk could be minimized when the track is thermally ablated during needle retraction [54]. By doing RFA the surrounding healthy liver parenchyma is preserved and therefore the procedure can be repeated several times for as long as the tumor remains localized to the liver and has not spread to loco-regional lymph nodes or to metastatic sites. The local recurrence rate at

the site of the ablation is low, ranging between 5-20% [55]. Overall RFA is a safe procedure that could take place in the outpatient setting.

The other commonly used local ablative therapy is local injection into the tumor of several agents. Ethanol is used worldwide however acetic acid has also been used with better results in regards to local recurrence compared to ethanol. The mechanism based on which this treatment approach is reasonable and effective (mainly in patients with cirrhosis) is that the liver tumor is softer compared to the surrounding cirrhotic liver. This allows for injection of large volumes of ethanol into the tumor with destruction of the cancer cells without diffusion into the rest of the liver. Similarly to the RFA the tumor should be small and ideally not exceeding 3cm. Typically up to 3 injection are required for the treatment of each given tumor. The local recurrence rate is estimated to be up to 15%. Based on a randomized trial that compared those two approaches it seems that RFA carries an improved survival compared to percutaneous ethanol injection [56].

1.2.7.4 Radiation

The liver has limited tolerance to radiation and therefore whole organ irradiation is not a treatment for liver cancer. The hepatic toxicity as a result of this is Radiation Induced Liver Disease (RILD), a clinical syndrome mimicking veno-occlusive disease following bone marrow transplantation, presenting with anicteric ascites, hepatosplenomegaly and elevated alkaline phosphatase, generally seen within 3 months following radiation[57]. Therefore whole-liver irradiation is used predominantly for palliation of symptoms such as pain. An alternative method to deliver radiation therapy to liver tumor is by hepatic arterial delivery of radioisotopes. The

most common radioisotope used in liver cancer is yttrium-90 (Y^{90}). Y^{90} is incorporated into stable glass (TheraSphere) or resin microspheres (SIRTex Medical).

1.2.7.5 Systemic Therapy

Systemic therapies for locally advanced and metastatic hepatocellular carcinoma involve chemotherapy, targeted therapy and immunotherapy. The role of adjuvant chemotherapy after surgery or orthotopic liver transplantation remains uncertain. Based on several studies and metanalysis there is no clear advantage in disease-free or overall survival for either adjuvant or neoadjuvant chemotherapy. A large number of controlled and uncontrolled clinical studies have been performed with the most commonly used chemotherapy drugs as single agents or in combinations. Despite the initial encouraging results the consensus is that no single agent or combination of agents given systemically could reproducibly lead to more than 25% response rated or has any effect in survival[58]. Single agent doxorubicin has been studied the most but even with a dose of 75mg/m2 the objective response rate is less than 20%. Lai et al showed a 10.6 weeks benefit with doxorubicin versus 7.5 weeks with supportive care in a small controlled clinical trial[59]. Fluoropyrimidines and specifically 5-fluorouracil (5-FU) when given in combination with leucovorin has a comparable response rate with acceptably low toxicity profile. There are several combination studies with cisplatin based therapies with moderate results in regards to response rate and no significant impact on overall survival.

Immunotherapy and specifically chemo-immunotherapy with interferon which is an immunomodulatory cytokine given in combination with cisplatin, 5-FU and doxorubicin (The PIAF regimen) was associated with a 26% objective response rate. However the toxicity is high.

Molecularly targeted therapies that are directed against specific deregulated pathways play an important role in the management of several cancers with one of the first ones being hepatocellular. Sorafenib is a multi-targeted orally active small tyrosine kinase inhibitor that inhibits the Vascular Endothelial Growth Factor Receptor (VEGFR) intracellular kinase pathway as well as the Raf kinase. Several early stage clinical studies were conducted however the drug got FDA approval after the results from the phase III SHARP trial that showed a survival benefit compared to best supportive care[60]. Ever since the use of sorafenib several other small molecule tyrosine kinase inhibitors have been used in multiple clinical trials without providing any additional survival advantage.

1.2.7.6 Regional Chemotherapy

In contrast to the dismal results of systemic therapy for patients with locally advanced, unresectable or metastatic HCC, a variety of agents given via the hepatic artery have activity in tumors confined to the liver. Transhepatic Artery ChemoEmbolization (TACE) showed promising results when doxorubicin or cisplatin was used in randomized controlled trials. Along with the chemotherapy agent, an embolizing agent such as lipiodol, gelatin (Gelfoam), starch (Spherex), microspheres and polyvinyl alcohol (Ivalon) have been used. The widespread use of some form of embolization in addition to chemotherapy has added to its toxicities. These include a frequent but transient fever occurring the night of the procedure, abdominal pain, anorexia in more than 60% of the patients, nausea, transient elevation of transaminases and increased ascites[61]. Interestingly it is not clear how to measure the response from TACE as the formal CT response criteria of oncologic partial responses are likely not adequate for patients with HCC. It appears that a loss of

vascularity seen on CT even without size change is also a reasonable index of loss of viability and thus tumor response to TACE [62].

1.3 Molecular Mechanism in Hepatocellular Carcinoma

A better understanding of the molecular pathways that are postulated to be involved in liver cancer pathogenesis will eventually lead to a better design of chemical compounds that could potentially target those. The most important liver cancer signaling pathways are described below.

1.3.1 JAK/STAT pathway.

In a wide variety of human diseases including hematologic malignancies and solid tumors the JAK/STAT (Janus-activated kinase/signal transducers and activators of transcription) pathway has been shown to be constitutively activated [63]. Interestingly, this pathway seems to link obesity with hepatocellular carcinoma via leptin signaling. In specific, it has been proposed that leptin-induced phosphorylation of ERK and AKT is dependent on JAK/STAT activation and promotes hepatocellular carcinoma growth, invasiveness, and migration [64].

1.3.2 The TGF-β / SMAD pathway.

A series of studies have shown that the TGF-β signaling pathway plays a significant role in liver cancer pathogenesis. Lee et al showed that invasive types of liver cancer tend to overexpress TGF-β and the levels are higher in liver tumors with portal vein thrombosis or extrahepatic metastasis compared to those without those features. In addition, high plasma TGF-β levels were inversely related to survival and in fact may be involved in rapid progression of liver cancer [65].

1.3.3 The c-Myc pathway.

15% of all genes are being controlled by the c-myc, which is a potent oncogene and a central regulator of many key functions in the cellular level. In liver tumors, myc is one of the most commonly activated oncogenes and it is correlated with poor prognosis [66]. Also, it has been shown that down-regulation of myc results in loss of the neoplastic properties of the tumor while overexpression can potentially induce liver cancer in animal models [67]. Furthermore, it is known that c-myc interacts with TGF-α and other oncogenic pathways leading to liver carcinogenesis [68].

1.3.4 The Wnt / β-catenin pathway.

Inappropriate activation of the Wnt/β-catenin pathway has been implicated in liver carcinogenesis. Specifically, 20-30% of liver cancer patients harbor mutations of the β-catenin genes [69]. Cross interaction between Wnt/β-catenin pathway and other signaling pathways such as that of TGF-β contributes to its dysregulation [70]. Furthermore, there is cross-interaction between the Wnt and the hepatocyte growth factor (HGF)/MET pathway. A recent study has shown that β-catenin associates with MET which is overexpressed in liver tumors and is correlated with poor prognosis [71].

1.3.5 The cell cycle and survival pathways (cyclins, p53, pRb, bcl-2).

Cyclins are a family of proteins that through the activation of the cyclin dependent kinases (CDKs) control the cell cycle [72]. A previous study has shown that cyclin G1 not only plays a critical role in liver cancer metastasis but also its expression and p-Akt levels are powerful predictors of poor prognosis in patients with liver cancer [73]. The p53 pathway is a major tumor suppressor pathway that plays a significant role in cell survival and proliferation in response to

telomere shortening and cell cycle arrest in response to oncogene activation [74]. At the stage of cirrhosis loss of p53 could cause chromosomal instability, proliferation of hepatocytes with dysfunctional telomeres and potentially initiation of liver carcinogenesis. Interestingly, p21, which is a downstream target of p53, is increased in cirrhosis but lost in liver cancer, supporting the above idea [75].

The p16/Rb pathway is another major tumor suppressor pathway that in at least 80% of hepatocellular carcinomas is disrupted. The postulated mechanism is methylation of the promoter of p16 leading to its down-regulation [76]. Similarly to p21, p16 is upregulated in cirrhosis (but not in liver cancer) and so inhibition of p16 is correlated with higher risk of liver carcinogenesis in patients with cirrhosis [77].

The members of the bcl-2 protein family are key determinants of apoptosis [78]. When chemical agents that exhibit BAD-like activity were used, the liver cancer cells became more sensitive to sorafenib, which is currently the only therapeutic drug against advanced liver cancer [79].

1.3.6 The RAS/RAF/MEK/ERK pathway.

Another important pathway is the Ras/Raf/MEK/ERK that is deregulated in liver cancer similarly to other malignancies. This signaling cascade is known to be involved in the proliferation and transformation of hepatocytes. Increased levels of ERK have been correlated with cancer progression [80]. Interestingly this pathway has also been involved in hepatitis C-infected liver cancer patients. Specifically, during HCV infection the Ras/Raf/MEK pathway is activated, which in turn attenuates the IFN-JAK-STAT pathway, resulting in stimulation of HCV replication which is one of the main causes of cirrhosis and potentially liver cancer [81].

1.3.7 The PI3K / PTEN / AKT / mTOR pathway.

It is well described that one of the key regulators of cell growth is the PI3K/PTEN/AKT pathway [51]. PTEN is a tumor suppressor that counters the activation of the PI3K (phosphatidylinositol 3-kinase). Activation of the PI3 kinase pathway increases the activity of the AKT kinase which phosphorylates mTOR (the mammalian target of rapamycin). Importantly, the mTOR kinase is activated in response to hypoxia and energy depletion and its activation has been correlated with radiotherapy and chemotherapy resistance [82] . In liver cancer PTEN is downregulated and it has been shown that PTEN suppression is associated with aggressive biological behavior and poor patient survival [83].

1.4 MicroRNAs

Great advances in molecular medicine have occurred over the last decade. Since the identification of DNA by Watson and Crick, the scientific community was studying the role of genes and their proteins. However the attention has now directed to non-protein coding genes that include the long non coding RNAs (lincRNAs) and the micro-RNAs (miRs).

1.4.1 What are the microRNAs.

We are at an exciting era in cancer research that started in the 1990s with the identification of a novel class of genes, the microRNAs, by Dr. Ambros who received in 2008 the Lasker award for that discovery [84]. They consist a new class of non-coding small RNAs, which regulate the expression of more than 30% of protein-coding genes at the post transcriptional and translational level. Each one can control hundreds of gene targets by translational repression, mRNA cleavage, and mRNA decay initiated by miRNA-guided rapid deadenylation [85,86]. They regulate

cell proliferation and apoptosis and function as oncogenes or tumor suppressors [87,88]. It is important to mention that so far they were considered to be negative regulators but recent studies showed that can also have positive effect on genes. More than 50% of microRNA genes are located in cancer-associated genomic regions or in fragile sites, suggesting that miRNAs may play a more important role in the pathogenesis of a limited range of human cancers than previously thought [89]. The major steps for cancer development include initiation, promotion, malignant conversion, progression, and metastasis [90]. Many factors influence the development of cancers through inhibition or promotion of tumor development. Actually there is a combined interaction of both tumor suppressors and cancer inducers genes, the so-called oncogenes. The unique expression profiles of different microRNAs in different types of cancers and at different stages in one cancer type suggest that microRNAs can function as novel biomarkers for disease diagnostics and may represent a new strategy for microRNA gene therapy [91].

1.4.2 MicroRNA biogenesis

The first micro RNA (lin-4) was found in 1993 and since then more than 700 microRNAs have been identified and being added in microRNA databases [84]. In order to understand the action of microRNAs we need to know how they are produced. The main characteristic is that they are single-stranded RNAs consisting of 20 to 25 nucleotides. MicroRNAs originate from genes that are called miRNA genes. Specifically, in the nucleus large RNA precursors (whom length vary from several tenths to more than 1000 nucleotides) are named primary microRNAs (pri-miRNAs) and transcribed by RNA polymerase II (Pol II) as well as Pol III with 5' cap and 3' poly A tails (**Figure 1**). These pri-miRNAs are recognized by a microprocessor complex which is composed of the nuclear RNase III Drosha together with its double-stranded RNA binding

domain (dsRBD) partner DiGeorge syndrome critical region 8 (DGCR8). The pri-miRNAs are cut into the miRNA precursors (pre-miRNAs) with an approximately 70 nucleotide stem-loop structure [92]. The pre-miRNAs with 2nd hairpin structure are then transported into cytoplasm by the transporter Exportin 5, and this process is RanGTP dependent. In the cytoplasm, the pre-miRNAs were further processed into the 19–24 nucleotide double-stranded miRNA miRNA* complex by another RNase III enzyme, called Dicer, together with its dsRBD partner TRBP [93,94]. Then, the mature miRNA sequences enter the RNA-induced silence complex (RISC) and target specific gene expression while the opposite strand miRNA* sequences are degraded by an unknown mechanism to date.

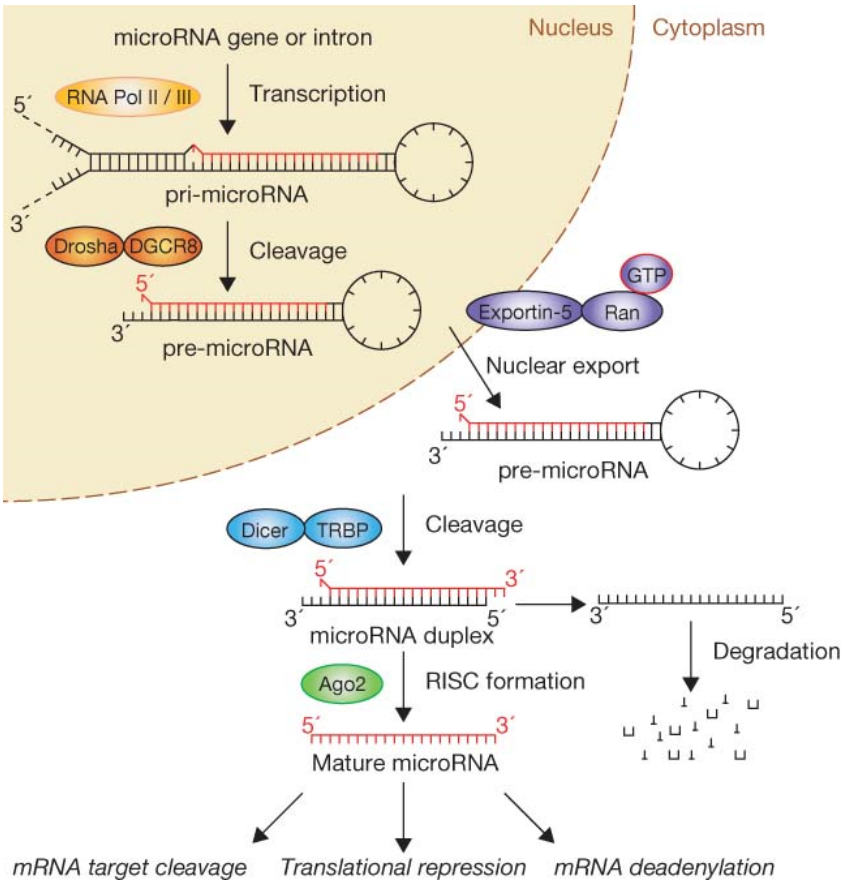

Figure 1. Mechanisms of MicroRNA Biogenesis (adjusted from Winter J et al. Nature Cell Biology, 11: 228-234, 2009).

1.4.3 The mechanism of action of microRNAs

The mechanism of action of microRNAs is fascinating and described below (**Figure 2**). MicroRNAs can bind with complementarity or imperfect complementarity to each strand of the double stranded RNA and depending on that can regulate the gene expression. There are three major currently-known mechanisms for miRNA-mediated gene regulation: translation repression, direct mRNA degradation and miRNA-mediated mRNA decay [95,96]. Which mechanism controls gene expression is entirely dependent on the degree of miRNA complementarity to their targeted mRNAs. MicroRNAs bind, in most cases, with imperfect complementarity to their targeted mRNAs and guide mRNA translation repression. However, there are also several miRNAs which directly degrade their targeted mRNAs. The exact mechanism for miRNA-mediated translation repression is still unknown but most likely miRNA-RISC complex inhibit the initiation and/or elongation of protein translation by interacting with various translation factors. Furthermore, it is shown that miRNAs mediate gene expression by guiding mRNA decay through de-adenylation and de-capping process of targeted mRNAs, which is completely different from normal translation repression and/or direct mRNA degradation [97]. It is well known that the 3' poly(A) tail and 5' cap are very important for mRNA stability and avoiding mRNA decay. When miRNAs guide the removal of the 3' poly(A) tail and 5' cap of the targeted mRNAs, these targeted mRNAs will be degraded by cellular enzymes. In a majority of cases, miRNAs bind to their targeted mRNAs at the 3' UTR with multiple sites. However, miRNAs targeted to the 5' UTR and/or the open reading frame (ORF) can also repress gene expression [98]. MicroRNAs interact with their targeted mRNAs primary through the six to eight nucleotides at the 5'end of miRNAs, which is perfectly bound to the targeted mRNAs. This region is called 'seed' sequence in miRNAs and is high conserved in a same miRNA family from

species to species [99]. The majority (61%) of miRNA genes are located at an intronic region of protein-coding genes; however can also be in regions of exons or intergenes. Interestingly, more than 50% of miRNA genes can be found in cancer- associated genomic regions or in fragile sites, suggesting that miRNAs play an important role in the pathogenesis of neoplasias[89].

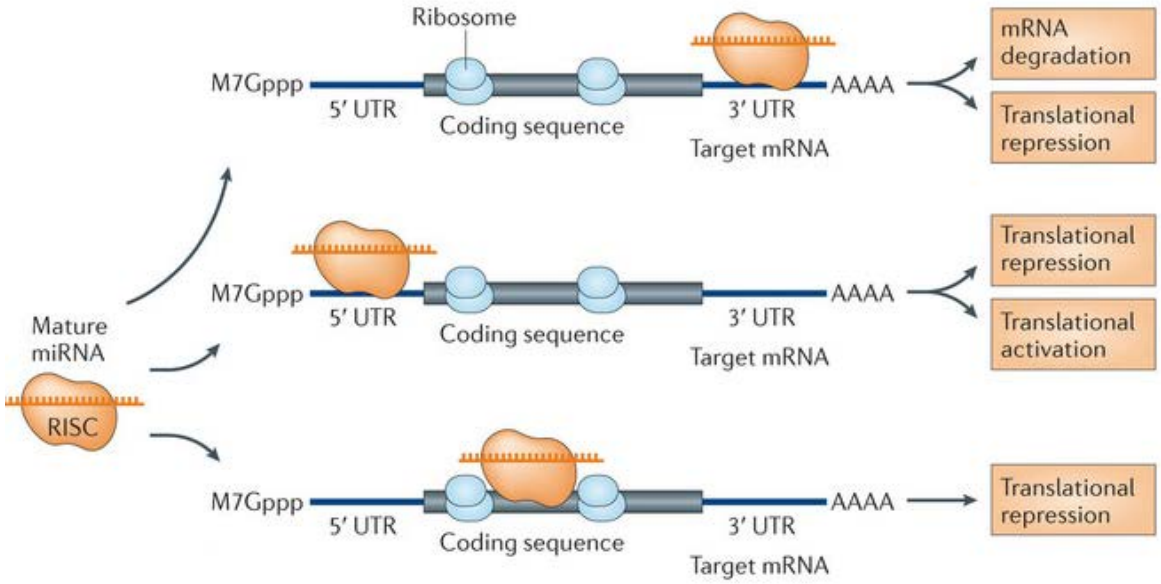

Figure 2. Mechanisms of Action of MicroRNAs (modified from Ling H et al. Nature Rev Drug Discovery, 12, 847-865, 2013).

1.4.4. The clinical implication of microRNAs

Several studies showed that miRNAs have a fundamental role in a great spectrum of diseases including but not limited to cardiovascular, autoimmune, metabolic diseases and certainly cancer.

1.4.4.1. The role of microRNAs in human diseases

Obesity is the disease of our days, is increasing in incidence especially in the western countries and is leading to insulin resistance and eventually diabetes. There is an increased need to understand glucose metabolism and slow down the rate of diabetes. Poy et al. showed that overexpression of miR-375 inhibited glucose-induced insulin secretion. In conversion, down-regulated miR-375 promoted insulin secretion. This suggests that miR-375 is a regulator of insulin secretion and may become a novel pharmacological target for the treatment of diabetes[100].

The number one risk of death remains the cardiovascular disease and hyperlipidemia is one of the major risk factors. Esau and colleagues studied the effect of miR-122 in lipid metabolism and observed the decrease in plasma cholesterol level and a significant improvement in liver steatosis by inhibiting miR-122 expression[101].

A new class of miRNA inhibitors which are known as antagomirs were used to knockdown miR-122. It was found that down-regulation of miR-122, significantly decreases the plasma cholesterol levels after four days of treatment, which suggests that miR-122 is a key regulator of cholesterol and fatty acid metabolism in the adult liver and a promising therapeutic target for metabolic diseases. Many miRNAs have also been identified in several viruses, like HIV, HBV,

HCV, Epstein Barr virus (EBV) and human cytomegalovirus (HCMV). Surprisingly, viruses are involved in miRNA production but the exact mechanism for virus-encoded miRNA biogenesis is still unclear. One important function of miRNAs is to control viral replication when the virus infects a cell and in that way to further control virus infection.

A liver-specific miRNA, the miR-122 (which was mentioned previously) modulates HCV RNA abundance and HCV replication. In a recent and very important study it was reported a significant loss (about 80%) of autonomously replicating hepatitis C viral RNAs by knocking down miR-122. However, studies with replication-defective RNAs demonstrated that miR-122 did not significantly influence mRNA translation or RNA stability, suggesting that miR-122 is likely to facilitate replication of the viral RNA [102].

There are thousands of studies that reveal a correlation between deregulated microRNAs and other human diseases and our main focus here is cancer.

1.4.4.2. The role of microRNAs in cancer.

Cancer is the first leading cause of death after cardiovascular diseases. There are five steps for the development of cancer which are initiation, promotion, malignant conversion, progression, and metastasis and it has been shown that microRNAs are involved in each one of them. In order to identify the exact role of micro RNAs in cancer pathogenesis, specific microRNAs were overexpressed or knocked down and the initiation and development of different types of malignancies were observed. Recognition of miRNAs that are differentially expressed between

tumor tissues and normal tissues may help to identify those miRNAs that are involved in human cancers and further establish the apparent pathogenic role of miRNAs in cancers.

In lung cancer one of the first identified microRNAs, miRNA let-7 was shown to control lung carcinogenesis, or at least play a critical role in the pathogenesis of this malignancy. Takamizawa et al. observed that the expression levels of let-7 were frequently reduced in both in vitro and in vivo lung cancer studies and reduced let-7 expression was significantly associated with shortened postoperative survival independently of disease stage [103]. Regarding breast cancer, Iorio et al. found that the miRNA expression patterns were significantly different between normal and neoplastic breast tissues; miR-125b, miR-145, miR-21, and miR-155 were significantly reduced in breast cancer tissues. They also observed that the expression of miRNAs was correlated with specific breast cancer bio-pathologic features, such as tumor stage, proliferation index, estrogen and progesterone receptor expression, and vascular invasion [104]. Colorectal neoplasia is also associated with alteration in miRNA expression. A recent study by Michael et al. identified 28 different miRNAs in colonic adenocarcinoma and normal mucosa, and found that the expression of two mature miRNAs, miR-143, and miR-145, was consistently reduced at the adenomatous and cancer stages of colorectal neoplasia [105]. Interestingly, a recent study showed that miR-145 and miR-21 play a critical role in regulating the growth of colon cancer stem cells and potentially their differentiation to colon cancer as well as progression to become resistant to chemotherapy [106].

1.5 Risk factor related microRNAs in liver oncogenesis.

As described previously there are various risk factors critical for liver cancer development and therefore it is important to identify potential links between these factors and specific microRNA pathways **(Table 9).**

Table 9. Liver cancer risk factors related microRNAs that are deregulated and affect relevant signaling pathways.

MicroRNA	Most Common Targets	Function
HCV RELATED		
MiR-122	Interaction with viral genome	HCV/HBV replication
MiR-100	Unknown (potential BAZ2A)	Unknown
MiR-10a	Unknown (potential HOXA1)	Unknown
MiR-193b	Mcl-1	Apoptosis, Chemoresistance
MiR-196	Bach-1	HCV gene expression
MiR-198	Unknown (potential FGFR1)	Unknown
MiR-145	MAP3K, MAP4K4	Cell growth, cell survival
HBV RELATED		
MiR-221	p27	Cell growth
	DDIT4	Cell survival
MiR-152	DNA methyltransferase 1	HBV DNA methyltransferase
ALCOHOL INDUCED		
MiR-155	unknown	TNFa production
Mir-212	Zonula occludens-1	Epithelial junctions
NASH INDUCED		
MiR-467b	Lipoprotein lipase	Insulin resistance
MiR-122	HMGCR, FAS, SREBP-2,SREBP-1C	Lipid metabolism

1.5.1. HCV-related microRNAs.

One of the most well studied microRNAs in liver cancer is miR-122 which has been found to increase both HCV replication and translation. Furthermore, there is inverse correlation between miR-122 expression levels and the severity of hepatic fibrosis and potentially cirrhosis due to

HCV. Therefore, targeting miR-122 may emerge as a strategy in the treatment of this infection [107]. MiR-199a is another candidate that could modulate HCV expression by down regulating the viral RNA replication [108]. Similarly, miR-196 down-regulates the expression of HCV and interestingly is upregulated in response to interferon which is one of the known treatments of active hepatitis C [109]. Many other microRNAs have been proposed to be deregulated in virus associated liver cancer. Specifically, miR-122, miR-100 and miR-10a are upregulated, whereas miR-198 and miR-145 are down-regulated in HCV-related hepatic tumors compared to normal tissues [110]. Regarding the HCV-infected liver cell lines, miR-193b is 5-fold up-regulated. MiR-193b targets Mcl-1, an anti-apoptotic protein that can modulate the response to sorafenib. Finally, microRNAs have been identified to play a role in HCV replication, such as miR-491 which could suppress HCV replication via the PI3 kinase/Akt pathway [111].

1.5.2 HBV-related microRNAs.

It has been shown that microRNAs regulate the activity of hepatitis B virus and in fact the virus itself uses its own viral microRNAs to regulate its gene expression [112]. On the other hand, another study showed that the expression of HBV-specific microRNAs could suppress HBV replication [113]. In addition, miR-221 has been identified to be downregulated in acute HBV infection, normally expressed in chronic HBV infection, and upregulated in liver cancer, indicating that it may be a key effector for progression of the disease [114]. Furthermore, miR-152 is frequently down-regulated and has a tumor suppressive role in HBV related liver cancer [115]. On the other hand, miR-122 is associated with a decreased replication of HBV [116].

1.5.3 MicroRNAs in alcohol induced liver disease.

Chronic alcohol consumption causes liver toxicity that leads to cirrhosis and eventually liver cancer. Bala et al., showed that alcohol increases miR-155 levels, via NF-κB, which then induces TNF-α [117]. Furthermore, another study described that alcohol consumption leads to overexpression of miR-212 [118]. Therefore it is believed that there is a potential link between microRNA deregulation and alcohol-induced liver disease.

1.5.4 MicroRNAs in Non Alcoholic SteatoHepatitis (NASH).

Non-alcoholic steatohepatitis which is well known as fatty liver is a common disease that is increasing in incidence due to the western diet and obesity. Previous studies have revealed deregulation of microRNAs targeting adipokines, cytokines and lipid metabolism pathways [119,120]. Specifically, it has been shown that inhibition of miR-122 potentially contributes to altered lipid metabolism and is implicated in the pathogenesis of NASH [121]. Furthermore, it has been described that miR-370 affects lipid metabolism and so fatty liver development by controlling the expression of miR-122 [122]. In addition, it has been shown that in high fat diet fed mice, miR-467b was down-regulated resulting in up-regulation of the hepatic lipoprotein lipase. That interaction is linked with insulin resistance which is one of the major causes of NASH [123]. Recently, miR-10b was found to play a role in lipid metabolism and in steatohepatitis via regulation of the PPAR-alpha expression [124]. Taken together all this information suggests the involvement of microRNAs in NASH-related liver oncogenesis.

1.6 MicroRNAs regulating essential signaling pathways in liver cancer.

There is a long list of microRNAs that are involved in signaling pathways implicated in liver cancer. Below we discuss the most important deregulated microRNAs and their role in liver oncogenesis (**Table 10**).

Table 10. Deregulated microRNAs and pathways in liver cancer

UP-REGULATED MICRORNAs			
MicroRNA	**Target Gene**	**Function**	**Deregulated Pathway**
MiR-18a	ESR1	Estrogen activity	Estrogen receptor
MiR-21	PTEN, TGFb1	Cell survival,invasion	PI3K/PTEN/mTOR/AKT
	SMAD3, TGFb2	migration,proliferation	
MiR-24	HNF4	transformation,	Cell cycle and survival
		migration, Invasion	JAK/STAT
MiR-25	Bim	Cell survival, apoptosis	Cell cycle and survival
MiR-30d	GNAI2	Migration, invasion,metastasis	RAS/RAF/MEK/ERK
MiR-151	RhoGDIA	Migration,invasion,metastasis	RAS/RAF/MEK/ERK
MiR-221	p27, p57, Bmf	Apoptosis,Cell growth,migration	Cell cycle and survival
DOWN-REGULATED MICRORNAs			
MicroRNA	**Target Gene**	**Function**	**Deregulated Pathway**
Let-7	c-myc, bcl-2	Cell proliferation,transformation	Cell cycle and survival
MiR1-1	c-met, FOXP1	Cell proliferation,transformation	Cell cycle and survival
	HDAC4	apoptosis, G2/M arrest	Histone modification
MiR-26a	cyclin D2,cyclin E2	Cell proliferation, apoptosis	Cell cycle and survival
MiR-29	mcl-1, bcl-2	Apoptosis, hypoxia	Cell cycle and survival
MiR-34a	c-met, bcl-2, CDK4	Cell survival, apoptosis,proliferation	Cell cycle and survival
MiR-101	mcl-1,fos	Apoptosis,hypoxia	Cell cycle and survival
MiR-122	bcl-w, cyclinG1	HBV/HCV replication	Cell cycle and survival
MiR-124	IL6R, CDK6,ezh2	EMT, transformation,apoptosis	JAK/STAT, histone modification
MiR-125b	p53	Apoptosis, cell proliferation	Cell cycle and survival
MiR-152	DNMT1	DNA methylation	DNA methylation
MiR-195	cyclin D1, E2F3, CDK6	Cell growth	Cell cycle and survival
MiR-199a	mTOR,, HIF1a	HCV RNA replication,cell proliferation	PI3K/PTEN/AKT/Mtor
MiR-223	stathmin	cell proliferation, cell growth	Cell cycle and survival
MiR-375	YAP, AEG-1	Cell proliferaton, invasion, apoptosis	Cell cycle and survival

1.6.1. Up-regulated microRNAs in liver cancer

miR-21

One of the most commonly described microRNA in the literature that has oncogenic role in most if not all tumors is microRNA-21. MiR-21 is highly overexpressed in most cancer types including liver cancer [95].

A possible role for miR-21 in the maintenance of the malignant transformation of hepatocytes, has also been proposed [125]. Inhibition of miR-21 increases the PTEN tumor suppressor directly and inhibits cell proliferation, migration and invasion [126]. Furthermore, miR-21 targets the TGF-β pathway and the inhibition of miR-21 results in overexpression of the ligands TGFB1, TGFB2 and SMAD3 [127].

miR-18a (miR-17-92 cluster)

MiR-18a, is also found to be overexpressed in HCC and belongs to the miR-17-92 cluster [128]. High miR-18a levels are associated with the female gender with a female to male ratio of 4.5. It has been described that miR-18a down-regulates ESR1 gene which encodes the estrogen receptor alpha (ERα). It is plausible that miR-18a suppresses the ERα and so diminishes the protective effect of the estrogens contributing to increased risk of liver cancer in women [129]. In addition, miR-17-92 cluster targets p21 which is a gatekeeper of the G1/S checkpoint and important for the cell cycle.

miR-24

It is well described and mentioned previously how inflammation could potentially lead to cancer and how inflammatory conditions such as cirrhosis increase the risk for hepatocellular carcinoma. Recently, we have shown that miR-24 is directly regulated by the IL6/STAT3 signaling pathway and is a part of a microRNA-inflammatory feedback loop circuit involved in liver cancer pathogenesis [130]. Specifically, we found that miR-24 has oncogenic function in the liver and its up-regulation increases the tumorigenicity and invasiveness of hepatocytes. MiR-24 overexpression results also in transformation of immortalized hepatocytes through direct regulation and suppression of the hepatocyte nuclear factor alpha (HNF4A) gene. In accordance to our study, miR-24 has been involved in HCV entry, replication and propagation [131].

miR-155

Another microRNA which is up-regulated in liver cancer and promotes cancer cell invasion is miR-155. Interestingly, in patients who underwent liver transplantation, as treatment for liver cancer, high levels of miR-155 were correlated with significantly decreased recurrence-free and overall survival [132]. MiR-155 targets directly the sex-determining region Y box 6 (SOX6), which up-regulates the p21waf1/cip1 in a p53-dependent manner, reducing liver cancer growth. Therefore, miR-155 through the SOX6/p21waf1/cip1 axis enhances liver tumorigenesis [133].

miR-25 (miR-106b-93-25 cluster)

MiR-25 is a member of the miR-106b-93-25 cluster and its up-regulation inversely correlates with Bim expression levels. Li et al., showed that inhibition of miR-106b-93-25 decreased liver cancer cell viability and anchorage independent growth [128]. This cluster of microRNAs has been

shown to target p21, which is a p53 target of the Cip/Kip family, and to abrogate TGF-β induced cell cycle arrest and apoptosis [134] [135]. It has been shown that miR-25 is elevated in up to 70% of patients with liver cancer.

miR-30d

MiR-30d is a microRNA that is amplified on chromosome 8q24 which is a common recurrent amplification region. It is up-regulated in liver cancer and has been shown to be involved in invasion and metastasis [136]. It has been shown recently, that miR-30d represses the direct and functional target Galphai2 (GNAI2) a G protein alpha subunit that inhibits adenylate cyclase activity. In a mouse model this interaction enhances migration, invasion of liver cancer cells and promotes intrahepatic and distal pulmonary metastasis.

miR-221-222 cluster

70% of liver cancer patients have overexpressed the miR-221-222 cluster that inhibits apoptosis through negative regulation of the CDK inhibitors p27 and p57 [137,138]. Pineau et al. described that overexpression of mir-221 via the m-TOR pathway stimulates the growth of tumorigenic murine hepatic progenitor cells [139]. A recent study, showed that the miR-221-222 cluster targets the tumor suppressors PTEN and TIMP3, leading to activation of the AKT pathway and metallopeptidases. Through that interaction, miR-221 induces TNF-related apoptosis-inducing ligand (TRAIL) resistance and enhances cellular migration [140]. On the other hand, silencing of miR-221 increases liver cancer cell death, by caspase 3 cleavage [138]. In addition, high levels of expression of the miR-221-222 cluster is correlated with poor prognosis [141].

miR-151

MiR-151 is located on chromosome 8q24 and is co-expressed with the focal adhesion kinase (FAK) and synergistically promotes liver cancer metastasis[142]. Furthermore miR-151 through direct regulation of the PhoGDP dissociation inhibitor (RhoGDIA), activates Racl, Cdc42, Rho GTPases and therefore promotes liver cancer cell migration and invasion [143].

1.6.2. Down-regulated microRNAs in liver cancer

Let-7

One of the first microRNAs that have been identified and studied in depth is let-7. The let-7 family plays significant role in different types of cancer and has been found to be down-regulated in liver cancer compared to cirrhotic liver [144]. Let-7 controls liver cancer growth through regulation of different target genes. One study has shown that let- 7 blocks liver cancer cell proliferation by down-regulation of c-Myc and upregulation of p16(INK4A)[145]. Furthermore, let-7 inhibits the bcl-2 expression and potentiates sorafenib-induced apoptosis [146]. In addition, let-7 is significantly suppressed in metastatic liver cancer and is correlated with poor survival [147]. Also, down-regulation of let-7 could lead to overexpression of miR-17-92 which has been shown to be essential for the malignant transformation of hepatocytes [148].

miR-122

As previously described microRNA-122 is the dominant microRNA in the liver and is down-regulated in liver cancer [102]. MiR-122 targets Bcl-w mRNA and miR-122 overexpression leads to

increased caspase-3 activity and induction of apoptosis [149]. It has been claimed that this phenomenon could be due to miR-122-mediated silencing of cyclin G1 [144].

miR-124

There is extensive amount of work and study done around this microRNA. MiR-124 has been found to be down-regulated in liver cancer through DNA hypermethylation, regulating directly CDK6 [150]. Low levels of miR-124 correlates with the aggressiveness of the tumor and poor prognosis. Overexpression of miR-124 *in vitro*, inhibits cell motility and invasion, while overexpression in mice suppresses intrahepatic and pulmonary metastasis. Furthermore, miR-124 binds to the 3'UTR of two oncogenes, the ROCK2 and EZH2 and inhibits their expression. It has been proposed that probably through this interaction, overexpression of miR-124 inhibits epithelial mesenchymal cell transition (EMT) [151] . In addition, we have recently demonstrated that systemic administration of miR-124 in mice suppresses hepatocellular carcinogenesis by inducing tumor specific apoptosis without any significant toxicity [130].

miR-26a

MiR-26a has been found to be down-regulated in liver cancer and even though predicts poor survival it was associated with favorable response to interferon therapy [152]. Interestingly, increased miR-26 levels are associated with increased patient survival [107]. Overexpression of miR-26a directly targets cyclin D2 and cyclin E2 expression levels and induces liver cancer cell cycle arrest. In mouse models, administration of miR 26a, by using adenoassociated virus (AAV)

as the delivery vector, blocked liver cancer cell proliferation, induced tumor specific apoptosis and suppressed disease progression without any significant toxicity [153].

miR-1-1

MiR-1-1 has been found to be down-regulated in liver cancer due to DNA hypermethylation in its promoter area and regulates directly the expression levels of c-met, FoxP1 and histone deacetylase 4 (HDAC4), leading to increased liver cancer cell proliferation. The same study has shown that overexpression of miR-1-1 in cancer cell lines causes G2/M arrest, decreases the colony formation ability and leads to apoptosis [154].

miR-29

MiR-29 is down-regulated in liver cancer targeting directly the anti-apoptotic Mcl-1 and Bcl-2 [155]. Low levels of miR-29 in liver cancer cells make them resistant to hypoxic conditions and apoptotic signals. On the other hand, enhanced miR-29 activity sensitizes liver cancer cells to chemotherapy [156].

miR-34a

MiR-34a is down-regulated in liver cancer and is directly regulated by the tumor suppressor p53 [157]. Loss of p53 could potentially fail to stimulate miR-34 expression, allowing unchecked proliferation and survival. C-met is targeted by miR-34a and Li et al. showed that the miR-34a decreased c-met induced phosphorylation of extracellular signal regulated kinases 1,2 and inhibited tumor cell migration and invasion [158]. Furthermore, miR-34a also regulates the

antiapoptotic proteins Bcl-2, N-myc as well as the cyclin E2 and cyclin-dependant kinase 4 (CDK4) which may allow proliferation when this microRNA has low levels of expression [159].

miR-101

MiR-101 is down-regulated in the majority of cancer tissues including liver cancer and it targets an anti-apoptotic member of the bcl-2 family, mcl-1 (myeloid leukemia cell 1). Overexpression of miR-101 sensitizes the cells to apoptosis, while miR-101inhibition enables the cells to evade apoptosis and to survive in hypoxic and nutrient depleted environments [156]. Furthermore, it has been shown that miR-101 targets the oncogene FOS, resulting in suppression of the Hepatocyte Growth Factor-induced invasion and migration [160]. In addition, in vivo delivery of miR-101 suppressed liver cancer formation and sensitized tumor cells to apoptosis after exposure to chemotherapeutic drugs possibly through targeting the epigenetic factor EZH2 [161]

miR-125b

MiR-125b expression is suppressed in liver cancer and it has been found that miR-125b overexpression suppresses liver cancer cell proliferation by decreasing Akt phosphorylation [162]. Furthermore, miR-125b targets p53 and overexpression of miR-125b suppresses apoptosis through negative regulation of p53, while knockdown of miR-125b induces apoptosis [163]. Down-regulation of miR-125b is associated with poor prognosis and shorter disease free survival of patients with liver cancer [162].

miR-152

MiR-152 is involved in hepatitis B related liver cancer and it is found to be downregulated in liver cancer. Specifically, Huang et al., described that miR-152 binds at the 3'UTR of the DNA methyltransferase 1 (DNMT1) resulting in a reduction of global DNA methylation. On the other hand, inhibition of miR-152 causes global DNA hypermethylation and specifically methylation of two tumor suppressor genes, E-cadherin (CDH-1) and glutathione S-transferase P1 (GSTP1) [115].

miR-195

MiR-195 is reduced in liver cancer and is part of the miR-15/16/195 family. MiR-195 up-regulation down-regulates the Rb-E2F signaling pathway by targeting cyclin D1, CDK6 and E2F3. Through these interactions miR-195 blocks the G1-S transition and suppresses liver tumorigenicity[164].

miR-199a-3p

MiR-199a is found to be down-regulated in liver cancer and plays an indirect role in liver carcinogenesis by regulating HCV infection. Specifically, miR-199a blocks HCV RNA replication by binding in the 5'NCR of the HCV [108]. Furthermore, the miR-199a-3p cluster targets the mTOR signaling pathway which is a regulator of cell proliferation. Restoration of miR-199a-3p levels results in cell cycle arrest, decreased invasion and increased sensitivity of the cells to doxorubicin in vitro. On the other hand, in vivo low levels of expression of this cluster in patient samples post tumor resection are correlated with significantly decreased time to recurrence, postoperatively [107]. Jia et al. demonstrated that miR-199a binds in the 3'-UTR of

HIF-1α. Therefore, through downregulation of HIF-1α miR-199a inhibits liver cancer cell proliferation *in vitro* and *in vivo* [165].

miR-223

MiR-223 is found to be down-regulated in liver cancer and targets stathmin, a microtubule regulatory protein. Overexpression of stathmin controls the S-phase of the cell cycle and increases proliferation. Interestingly, it has been demonstrated that re-expression of miR-223 leads to constant inhibition of the cell viability [166].

miR- 375

MiR-375 is down-regulated in liver cancer and targets directly the Hippo-signaling effector yes-associated protein (YAP), a potent oncogene, controlling liver cancer cell proliferation and invasion [167]. Overexpression of miR-375 in liver cancer decreased cell proliferation, clonogenicity, migration, invasion and also induced G1 arrest and apoptosis. More recently, it has been shown that miR-375, by targeting the astrocyte elevated gene-1 (AEG-1), is able to suppress liver cancer cell growth *in vitro* and *in vivo* [168].

1.7 Aim of our Research

The aim of this proposal is to understand the various steps leading to hepatocellular carcinogenesis by using the functions of microRNAs as the tools. Specifically we would like to investigate the role and functionalities of microRNAs and their downstream pathways in this type of cancer. Our approach is applying novel high throughput technologies such as microRNA microarray analysis and functional microRNA library screen followed by integration of these data using novel bioinformatic algorithms. Specifically, we performed 1) microRNA profiling in hepatocellular carcinomas and normal tissues in order to identify the differentially expressed microRNAs between normal and cancer liver tissues; 2) a functional microRNA library screen to identify the functionally important microRNAs in HCC; 3) integration of microRNA profiling and library screen data to identify microRNAs with functional importance and clinical relevance; 4) verify the importance of identified microRNAs in other hepatocellular cancer cell lines; 5) identify the down-stream targets and signaling of the identified microRNAs in HCC. All the aforementioned steps will be described in detail in the specific part.

2. SPECIFIC PART

2.1 Materials and Methods

RNA analysis from HCC and liver control samples

Initial analysis was started from human tissues. Specifically we extracted RNA from 24 Fixed-Formalin- Paraffin-Embedded (FFPE) HCC samples and 14 liver control (adjacent non-tumor) tissues. RNA was isolated using Trizol, according to manufacturer's instructions (Invitrogen). The specimens were obtained after consenting patients, right prior to their surgeries at the Department of Surgery at Stanford University, CA. The scientific protocol, as well as the consent form, were approved by the Ethics Committee of the Stanford University Medical School.

MicroRNA library screen

Library screen is a powerful tool that allows us to study and identify special properties of the cell lines and sets of genes that are functionally involved in carcinogenesis. Specifically we used SNU-449 liver cancer cells that were plated in 96-well plates in three triplicates and transfected with a microRNA library consisting of 316 microRNA mimics and 2 negative control microRNA mimics (100 nM) (Dharmacon Inc). The transfection dose of 100nM for the microRNA mimics was detected through control experiments performed to identify the maximum dose without any cytotoxic effects. At 48 hours post-transfection, SNU-449 cell invasiveness was evaluated in Boyden chamber invasion plates. Assays were conducted according to manufacturer's protocol, using 2% FBS as a chemoattractant. Invading cells were fixed and stained with 0.1% crystal violet, 24 hours post seeding. The cells that migrated through the filter were quantified by counting the entire area of each filter. MicroRNAs that affected >2-fold (50%) SNU-449 invasiveness relative to microRNA negative control treated SNU-449 cells were considered as positive hits.

Invasion assay

Invasion assay is one of the key techniques to study the principal properties of carcinogenesis. We performed invasion assays in SNU-449 cells 24 hours after transfection with miR-9 or anti-miR-9 and their respective controls. Invasion of matrigel has been conducted by using standardized conditions with BD BioCoat Matrigel invasion chambers (BD Biosciences). The Boyden chamber has an 8μm-pore membrane in the top side. Assays were conducted according to manufacturer's protocol in which 2% FBS was used as the chemoattractant. Non-invading cells on the top side of the membrane were removed, while invading cells were fixed and stained with 0.1% crystal violet, 24 hours post-seeding. The cells that migrated through the filter were quantified by counting the entire area of each filter, using a grid and an Optech microscope at a 20X magnification. The experiment was performed in triplicates and the statistical significance was calculated using Student's t test.

Real-time PCR analysis

Real-time PCR is a principal technique that permits the identification of specific, amplified DNA fragments using analysis of their melting temperature (T_m). The method used is usually PCR with double-stranded DNA-binding dyes as reporters and the dye used is usually SYBR green. The DNA melting temperature is specific to amplified fragment. In our work, quantitative real-time RT-PCR was performed to determine the expression levels of miR-9, miR-21 and miR-224 in 24 human HCC (stage I n=5; stage II n=9; stage III n=6; stage IV n=4) and 11 liver control tissues. RNA was isolated using Trizol, according to manufacturer's instructions (Invitrogen). Real-time RT-PCR was assessed on a CFX384 detection system (BioRad) using the Exiqon PCR primer sets according to manufacturer's instructions. MicroRNA expression levels were normalized to the levels of U6 small nuclear snRNA (203907, Exiqon). Normalized miRNA levels were quantified relative to the levels of a given control tissue. Real-time PCR was employed to determine the expression levels of CDH1, PPARA, vimentin and PDK4. Reverse transcription was carried out using the Retroscript Kit (AM1710, Applied Biosystems). Real-

time PCR was carried out using the IQ SYBR Green Supermix (170-8882, BioRad). Gene expression levels were normalized to the levels of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. Normalized gene expression levels were quantified to the respective control. The sequences of the primers used are the following:

CDH1-F: 5'-TGAAGGTGACAGAGCCTCTGGAT-3'

CDH1-R: 5'-TGGGTGAATTCGGGCTTGTT-3'

PPARA-F: 5'-GGCGAGGATAGTTCTGGAAGC-3'

PPARA-R: 5'-CACAGGATAAGTCACCGAGGAG -3'

Vimentin-F: 5'-CCAAACTTTTCCTCCCTGAACC -3'

Vimentin-R: 5'-GTGATGCTGAGAAGTTTCGTTGA -3'

PDK4-F: 5'-CCCCGAGAGGTGGAGCAT-3'

PDK4-R: 5'-GCATTTTCTGAACCAAAGTCCAGTA-3'

Colony formation assay

Colony formation assay is an assay based on the principle that certain proteins when expressed stably cause either cell cycle arrest or cell death, hence reduction in colony number. The assay should be stopped when the colonies are clearly visible even without looking under the microscope. In our experiment SNU-449 and HepG2 liver cancer cell lines were transfected with miR-9 or anti-miR-9 and their respective controls. Then, triplicate samples of $2x10^5$ cells from each cell line were assayed for colony formation using the CytoSelect Cell Transformation kit (Cell Biolabs, Inc). The number of colonies were counted after seven days.

Cell Growth assay

The CellTiter-Glo Luminescent Cell Viability Assay is a homogeneous method of determining the number of viable cells in culture, based on quantitation of the ATP present, an indicator of metabolically active cells. The homogeneous assay procedure involves adding the single reagent (CellTiter-Glo Reagent) directly to cells cultures in serum-supplemented medium. For our experiment SNU-449 and HepG2 liver cancer cell lines were transfected with miR-9 or the respective control and plated on a 96-well plate ($5x10^3$ cells/well). 48 and 72 hours later, cell growth was assessed using the Cell-Titer Glo Luminescence Cell Viability Assay (Promega).

Liver tumor sphere formation assay

A tumor sphere is a sold, spherical formation developed from the proliferation of one cancer stem/progenitor cell. There tumor spheres are easily distinguished from single or aggregated cells as the cells appear to become fused together and individual cells cannot be identified. Cells are grown in serum-free, non-adherent conditions in order to enrich the cancer stem/progenitor cell population as only cancer stem/progenitor cells can survive and proliferate in this environment. This assay can be used to estimate the percentage of cancer stem/progenitor cells present in a population of tumor cells. In our work, we performed the sphere formation assay. Specifically, SNU-449 liver cancer cell lines were transfected with miR-9 or anti-miR-9 were plated in ultra-low attachment plates (Corning), 24 hours post-transfection and were grown in DMEM F12 (Invitrogen) medium supplemented with B-27 (Gibco), bFGF and EGF in the culture medium containing 1% methyl cellulose to prevent cell aggregation. The number of spheres was evaluated six days post plating.

3’UTR luciferase assay

Firefly luciferase is commonly utilized as a reporter assay to evaluate the transcriptional activity in different cellular types. The most common application of luciferase reporter gene assay is to examine the regulation of the transcriptional activity of genes by transcription factors, through binding in their promoter areas. Recently, this assay has also been adapted for testing the effect of microRNA-mediated, post-transcriptional regulation on their direct target genes. This is achieved by engineering a luciferase gene construct containing the predicted microRNA seed sequence from the target gene (often located in the 3′-UTR). In our experiments, SNU-449 cells were transfected with a firefly luciferase reporter gene construct containing the 3’UTR of CDH1 (cat. no 25038, Addgene) or PPARA (cat. no HmiT054001-MT06, Genecopoeia). The constructs harbored the seed sequence of miR-9 (wildtype) or had a deletion of this sequence (miR-9 mutant). At 24 hours, they were transfected with miR-9 or miR-control and at 48 hours luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega).

2.2 Statistical Analysis

All experiments were performed in triplicate unless otherwise stated. Statistical analyses were performed with the use of Origin software, version 8.6. Student's *t*-test was used to examine the statistical difference in miR-9 expression between control and HCC tissues. The correlation significance was determined by means of Spearman and Pearson correlation analyses. A *P*-value of 0.05 or less was considered statistically significant.

2.3 Results

Strategy for the identification of functional and clinically relevant microRNAs in hepatocellular (HCC) oncogenesis.

It is well known that the identification of microRNAs which are differentially expressed between HCC and control tissues cannot predict if any of these microRNAs are functionally important in HCC pathogenesis. On the other hand, the identification of microRNAs affecting liver cancer cellular properties does not always suggest that these microRNAs would have any human relevance. The aim of our study was to identify microRNAs that have clinical relevance and they are differentially expressed in HCC tumors relative to control tissues and at the same time to have be functional important in HCC oncogenesis, by affecting HCC cellular properties. Thus, we have developed an experimental strategy, aiming to reveal the microRNAs that have both functional and human relevance in HCC (**Figure 3A**). Specifically, we followed a dual experimental approach by performing first a high-throughput microRNA screen in SNU-449 liver cancer cells and secondly, evaluated the expression levels of the microRNAs derived from this screen in human liver cancer and control tissues.

Identification of microRNAs regulating HCC invasiveness by performing a human microRNAome library screen in liver cancer cells.

Initially, we were interested in identifying the top microRNAs that were functioning as activators or suppressors of HCC invasiveness. To address this question, we performed a microRNA library screen in SNU-449 liver cancer cells. Specifically, we transfected a library of 316 microRNAs and two microRNA negative controls (miR-NC) and 48h post transfection, SNU-449 cell invasiveness was measured by performing a cell invasion Boyden chamber assay (**Figure 3B**). MicroRNAs that induced >2-fold SNU-449 invasiveness were characterized as microRNA invasion inducers and the microRNAs that suppressed >2-fold SNU-449 invasiveness were named as microRNA invasion suppressors (**Figure 3C**). Our screen revealed

five microRNAs (miR-9, -224, -21, -24, -27a) as HCC invasion inducers and 23 microRNAs (miR-29a, -145, -29b, -507, -26a, -122a, -375, -195, -203, -26b, -199b, -125a, -223, -1, -101, -199a, -124a, -125b, let-7b, let-7a, miR-148a, -152, -148b) as HCC invasion suppressors. Overexpression of miR-9 was found to be the top inducer of SNU-449 cell invasiveness (**Figure 3D**), a finding that has not been described in the literature in liver or other cancer types. Therefore it attracted our interest as a potential druggable target.

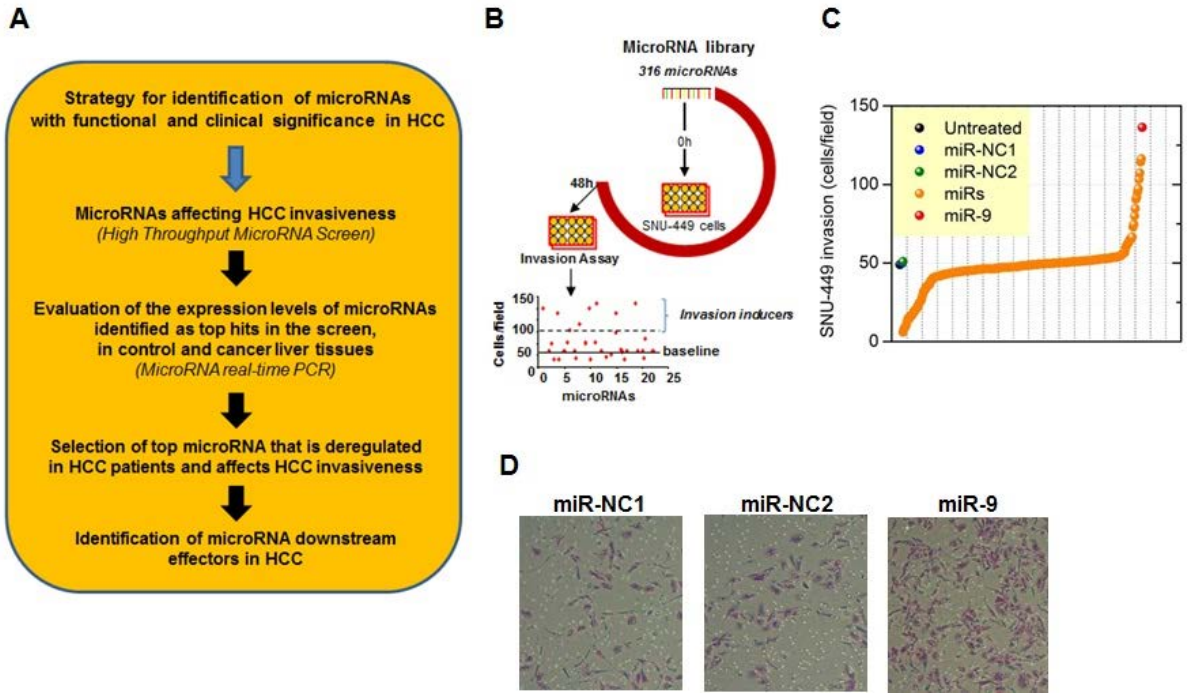

Figure 3. High-throughput screening identifies microRNAs that control HCC invasiveness. (**A**) Steps followed for identification of microRNAs with both functional and clinical significance in HCC. (**B**) Strategy workflow: A library of 316 microRNAs was transfected in SNU-449 liver cancer cells and their invasiveness was measured 48h post transfection in Boyden chamber invasion plates. (**C**) Screen data plotted as different microRNAs transfected in SNU-449 cells (x-axis) and their invasiveness (cells/field) compared to scrambled sequence controls (no effect, value=50) (y-axis). The red circle represents miR-9, while the blue and yellow circles

the microRNA negative controls (miR-NC1, miR-NC2). (**D**) SNU-449 cells stained with crystal violet in BioCoat Matrigel invasion chambers after treatment with miR-NC1, miR-NC and miR-9. Invading cells were fixed and stained with 0.1% crystal violet, 24 hours post-seeding. The cells that migrated through the filter were quantified by counting the entire area of each filter, using a grid and an Optech microscope at a 20X magnification.

Expression levels of microRNAs, acting as invasion inducers, in HCC patient tissues.

As aforementioned, due to the fact that we were interested in studying a microRNA that could be targeted by a microRNA inhibitor that potentially could be used in the human clinical setting, we focused our interest on the microRNAs that acted as inducers of HCC invasiveness. The screen above revealed that the top three microRNAs that were statistically significant inducers of liver cancer cell invasiveness were miR-9, miR-224 and miR-21. Thus, we evaluated their expression levels in 24 HCC tumors and 11 liver control tissues by real-time quantitative PCR analysis. MiR-9 was found to be 6.5-fold up-regulated in HCC relative to control tissues (**Figure 4A**) and miR-21 expression levels were increased 4.4-fold in HCC relative to controls (**Figure 4B**). In addition, miR-224 was found to be 6.4-fold up-regulated in HCC relative to controls (**Figure 4C**).

Following that, we have examined if there is any correlation between miR-9, miR-21 and miR-224 expression levels and HCC tumor stage. MiR-9 levels were found to increase during HCC progression (**Figure 4D**), having lower levels in early stages (stage I) and increasing until late stages (IV). MiR-21 expression was statistically different between stage I and II HCC tumors, while miR-224 expression was not statistically different between different HCC tumor stages (**Figure 5**). Taken together, these data suggest that miR-9 levels correlate with HCC disease progression and aggressiveness.

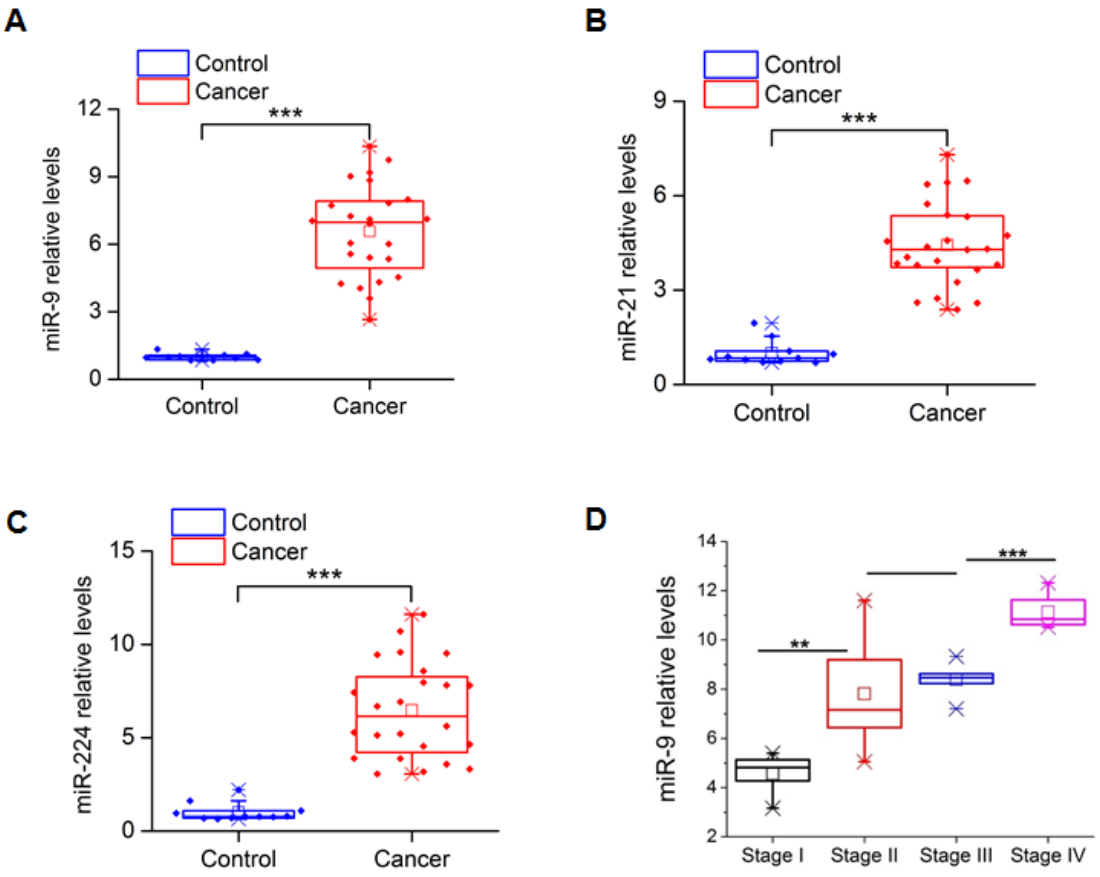

Figure 4. Relative microRNA expression levels in HCC and liver control tissues. (**A**) MiR-9, (**B**) miR-21 and (**C**) miR-224 expression levels in 24 HCC tumors and 11 control liver tissues assessed by real-time RT-PCR analysis. (**D**) MiR-9 expression levels in different stages of HCC tumors relative to controls. Data are represented as mean ± SE. ***P<0.001, in comparison to control.

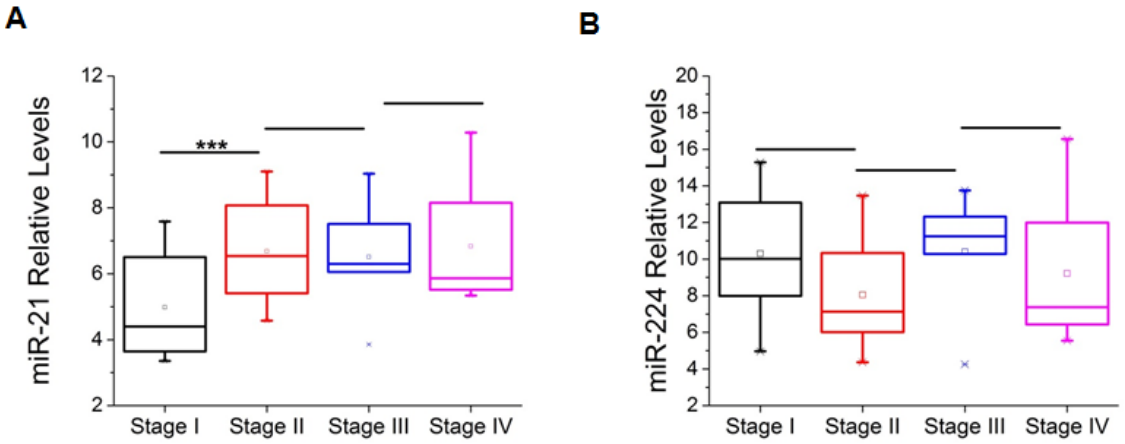

Figure 5. MicroRNA expression and HCC tumor staging. Relative **(A)** miR-21 and (**B**) miR-224 expression levels in HCC tumors in different stages (I, II, III, IV) assessed by real-time RT-PCR analysis and normalized to control liver tissues. Data are represented as mean ± SE. ****P*<0.001, in comparison to control.

The next step was to integrate the microRNA library screen and HCC tissue microRNA profiling data. This analysis revealed that miR-9 is the microRNA that has the highest ability to induce HCC invasiveness, it is highly expressed in HCC tumors and its expression correlates with HCC tumor stage, suggesting both its functional and human relevance in HCC.

MiR-9 is an inducer of HCC cancer cell properties.

As described above, we identified that miR-9 expression is increased HCC tumors in comparison to controls. To evaluate the oncogenic potential of miR-9 activity in HCC, we performed a series of cancer cell assays, by overexpressing miR-9 in SNU-449 and HepG2 liver cancer cell lines (**Figure 6A, Figure 7A**). The first step was to examine whether miR-9 affects liver cancer cell growth properties. Specifically, we transfected liver cancer cells with a miR-9 mimic or a microRNA negative control and evaluated their cell growth. MiR-9 was overexpressed in SNU-449 and HepG2 liver cancer cells and the total cell number was measured 48h and 72h post-transfection (**Figure 6B, Figure 7B**). Interestingly, we found that miR-9 induced liver cancer cell growth in both cell lines, more significantly 72h post transfection. Secondly, we were interested in understanding how miR-9 could potentially affect the invasiveness in liver cancer. Therefore we repeated the aforementioned transfection experiments with miR-9 mimic and control in order to study miR-9 effects on HCC invasiveness. We observed that miR-9 overexpression induced SNU-449 invasiveness (**Figure 6C, Figure 7C**), consistent with our primary microRNA library screen analysis. Furthermore, miR-9 overexpression induced ~2.3-fold HepG2 cell invasiveness, revealing that the effects of miR-9 on liver cancer cell invasiveness are not SNU-449 cell line specific. The next cancer property that we were eager to study was the role of miR-9 on soft agar colony formation and actually we identified that miR-9 overexpression induced significantly the ability of both SNU-449 and HepG2 cells to form colonies in soft agar (**Figure 6D, Figure 7D**). Finally, due to the fact that miR-9 may function as an oncogene, we examined its ability to regulate liver tumor sphere formation. We found that miR-9 overexpression increased the ability of SNU-449 cells to form spheres in suspension (**Figure 6E**). Taken together, these functional assays suggest that miR-9 plays an oncogenic role in HCC, affecting both cancer cell proliferation and invasiveness rates as well as colony and sphere formation.

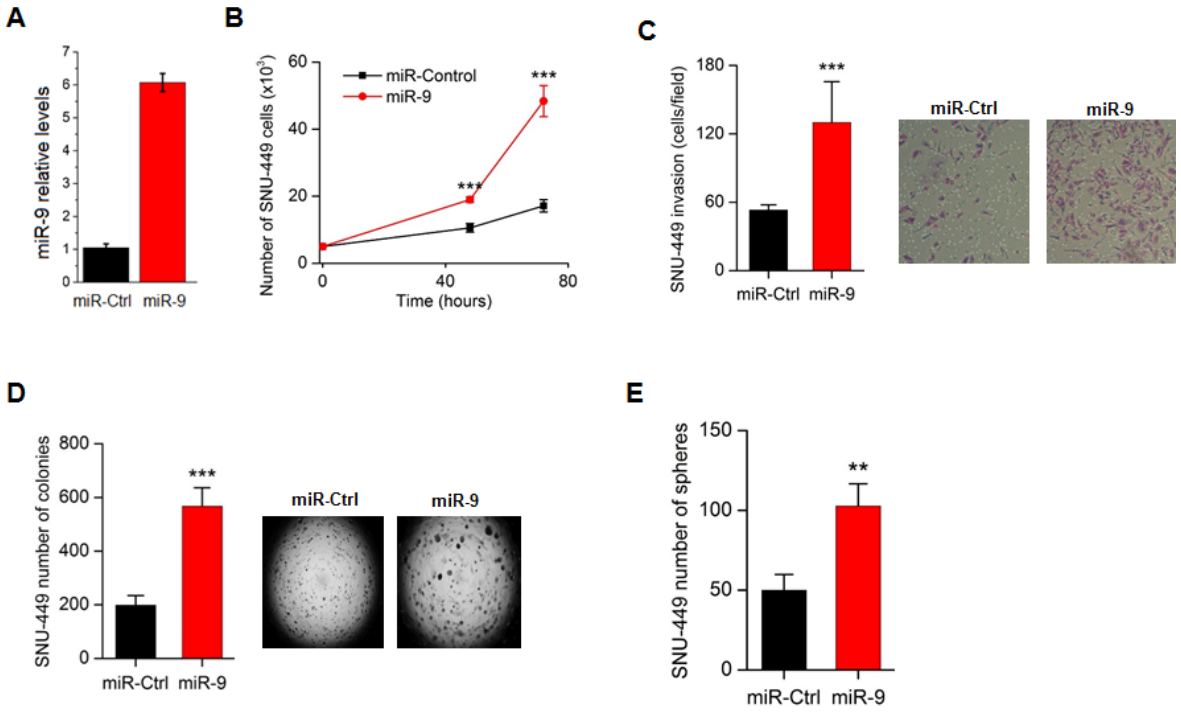

Figure 6. Effects of miR-9 overexpression on liver cancer cellular properties. (**A**) Relative miR-9 expression levels in SNU-449 cells after transfection with miR control or miR-9, 48h post-transfection. (**B**) Cell growth of SNU-449 liver cancer cells transfected with miR negative control (miR-Control) or miR-9, 48h and 72h post-transfection. (**C**) Invasion of SNU-449 after transfection with miR negative control (miR-Control) or miR-9, 48h post-transfection. (**D**) Soft agar colony assay in SNU-449 overexpressing miR-9 or miR-Control. (**E**) Effects of miR-9 overexpression on the number of SNU-449 liver tumor spheres. All data are represented as mean ± SE. ***P<0.001, **P<0.01.

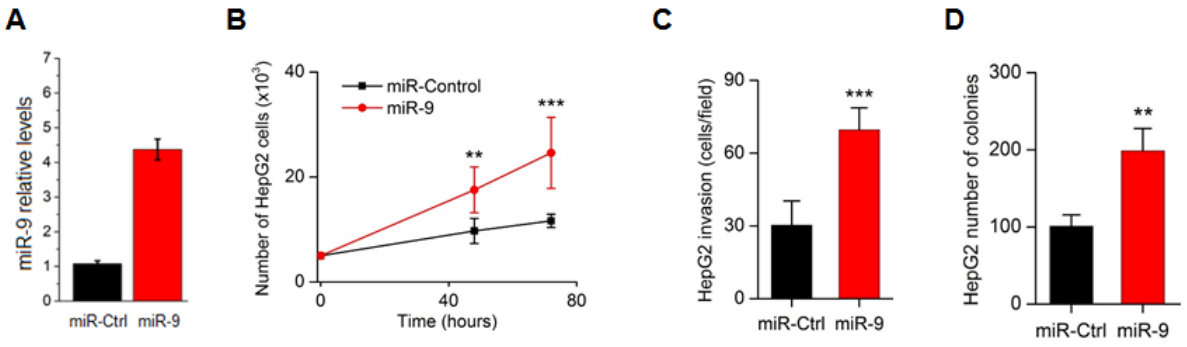

Figure 7. Effects of miR-9 overexpression on HepG2 cancer properties. (**A**) Relative miR-9 expression levels in HepG2 cells after transfection with miR control or miR-9, 48h post-transfection. (**B**) Cell growth of HepG2 liver cancer cells transfected with miR negative control (miR-Ctrl) or miR-9, 48h and 72h post-transfection. (**C**) Invasion of HepG2 after transfection with miR negative control (miR-Ctrl) or miR-9, 48h post-transfection. (**D**) Soft agar colony assay in HepG2 cells overexpressing miR negative control (miR-Ctrl) or miR-9. All data are represented as mean ± SE. ****P*<0.001, ***P*<0.01.

PPARA and E-cadherin (CDH1) as direct downstream targets of miR-9 in HCC.

Understanding the role of a specific microRNA in oncogenesis, it is essential to perform a comprehensive analysis and identify its direct gene targets and the signaling pathway(s) regulated by this microRNA. Here we were interested in examining the downstream gene effectors of miR-9 oncogenic activity in HCC. There are several bioinformatics programs that are publically available and each of these programs uses different parameters for the identification of the microRNA-direct gene targets. The main program that has been used

extensively in the literature and has been validated experimentally is the TargetScan algorithm (www.targetscan.org). Bioinformatics analysis revealed that miR-9 has very strong and highly conserved binding sites on the 3' untranslated regions (UTRs) of PPARA and CDH1 genes. Specifically, miR-9 has sequence complementarity in the position 7624-31nt of the 3'UTR of PPARA and also in the position 1327-33nt of the 3'UTR of CDH1 (**Figure 8A**). To examine the direct interactions between miR-9 and these potential downstream direct targets, we performed 3'UTR luciferase assays. MiR-9 was overexpressed in SNU-449 cells that were co-transfected with a construct harboring the 3'UTR of PPARA or CDH1 under luciferase activity. We found that miR-9 overexpression suppressed both CDH1 and PPARA 3'UTR luciferase activities, having a stronger effect on CDH1 (**Figure 8B**). Mutation of the miR-9 binding sites in the 3'UTR PPARA and CDH1 luciferase vectors abolished the suppressive effects of miR-9. These data validate at the molecular level of the direct interactions between miR-9 and PPARA or CDH1 genes and contributing in the identification of this signaling pathway. Following that, we examined the effects of miR-9 on CDH1 mRNA expression levels. We initially performed transfection with miR-9 mimic and miR negative control and showed that overexpression of miR-9 suppressed significantly CDH1 mRNA levels.

In the same context, having a similar experimental set up, we transfected the liver cancer cell lines with the antisense-miR-9 or the antisense-miR-control and evaluated CDH1 mRNA levels by real-time PCR analysis. Interestingly, we found that miR-9 overexpression resulted in up-regulation of CDH1 mRNA levels, in both SNU-449 and HepG2 liver cancer cells (**Figure 8C**). Due to the fact that CDH1 is an epithelial marker gene [169] and its loss has been correlated with epithelial mesenchymal transition (EMT), we examined the expression levels of the mesenchymal marker [170], vimentin which is known to be up-regulated during EMT, increasing cellular invasiveness phenotype. Real-time PCR analysis showed that miR-9 overexpression increased significantly vimentin mRNA levels (**Figure 8D**). In addition, miR-9 overexpression reduced PPARA mRNA levels in SNU-449 cells (**Figure 8E**). To further validate the miR-9/PPARA interaction, we examined PDK4 expression levels after miR-9 overexpression in liver

cancer cells. PDK4 is a known downstream direct target of the PPARA transcription factor in hepatocytes [171,172]. MiR-9 overexpression resulted in ~50% reduction of PDK4 mRNA levels, assessed by real-time PCR analysis (**Figure 8F**). Previous studies have identified a positive correlation between E-cadherin and the PPARA signaling pathways [173,174]. Therefore, we inhibited PPARA expression levels using a siRNA against PPARA (siPPARA) in SNU-449 and HepG2 cells and assessed levels of CDH1 mRNA by real-time PCR. Inhibition of PPARA resulted in >60% reduction in CDH1 mRNA expression levels in both cell lines (**Figure 8G**).

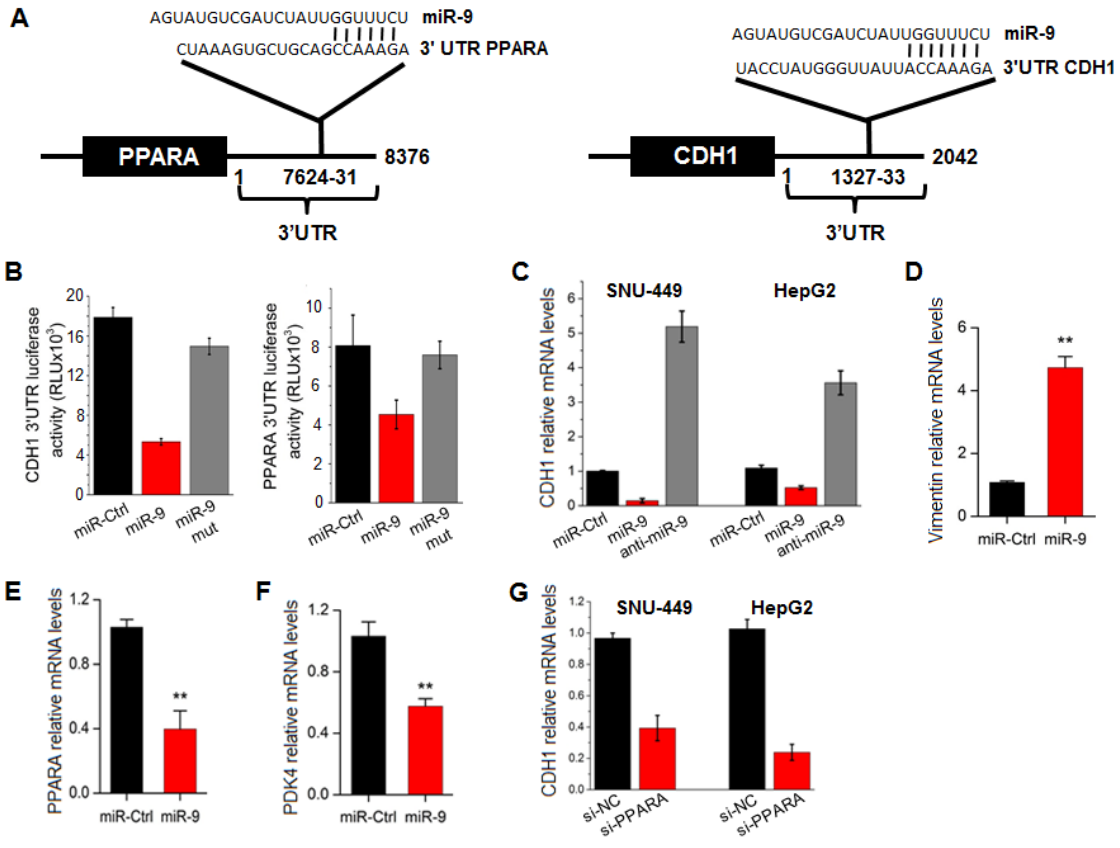

Figure 8. CDH1 and PPARA as direct targets of miR-9 in HCC. (**A**) Sequence complementarity between miR-9 seed sequence and the 3'UTRs of PPARA and CDH1. (**B**) CDH1 and PPARA 3'UTR luciferase assay activity in SNU-449 cells transfected with miR-Ctrl or miR-9, 48h post-transfection. MiR-9 sequence was wildtype or mutated (miR-9 mut). (**C**)

CDH1 mRNA levels in SNU-449 and HepG2 cells transfected with miR-9 or anti-miR-9, 48h post-transfection, assessed by real-time RT-PCR. (**D**) Vimentin, (**E**) PPARA and (**F**) PDK4 mRNA levels in SNU-449 cells transfected with miR-9, 48h post-transfection, assessed by real-time PCR. (**G**) CDH1 mRNA levels in SNU-449 and HepG2 cells transfected with an siRNA against PPARA (siPPARA) or an siRNA negative control (siCtrl), 48h post-transfection. All data are represented as mean ± SE. ****P*<0.001, ***P*<0.01, **P*<0.05.

Taken together, these data suggest that miR-9 regulates CDH1 expression directly through binding to its 3'UTR and indirectly by controlling PPARA expression. PPARA inhibition resulted in suppression of CDH1 mRNA levels, while CDH1 inhibition, by using an siRNA against CDH1, did not affect PPARA mRNA levels (**Figure 9**), suggesting that there is not a bi-directional regulation between PPARA and CDH1.

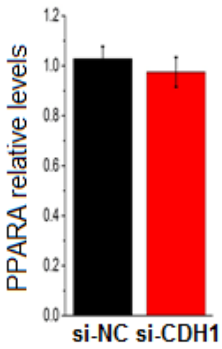

Figure 9. PPARA relative mRNA levels after CDH1 inhibition in SNU-449 cells. PPARA mRNA levels were measured by qPCR analysis in SNU-449 cells transfected with an siRNA negative control (si-NC) and an siRNA against CDH1 (si-CDH1), 48h post-transfection.

Suppression of the miR-9 signaling pathway on HCC cell properties.

To evaluate the therapeutic potential of miR-9 in HCC oncogenesis, we used an anti-sense miR-9 (anti-miR-9) and performed a series of experiments. First, we found that miR-9 inhibition suppressed significantly the ability of SNU-449 cells to form colonies in soft agar (**Figure 10A**), reduced their invasiveness (**Figure 10B**) and also their ability to form liver tumor spheres (**Figure 10C**). All these data reveal the therapeutic potential of targeting miR-9 in liver cancer. To further evaluate these findings, we examined the effects of PPARA inhibition on liver cancer cells. We found that inhibition of PPARA expression, by a siRNA (siPPARA), induced the ability of SNU-449 cells to form colonies in soft agar (**Figure 10D**) and increased their cellular invasiveness (**Figure 10E**), suggesting that PPARA has a tumor suppressive function in HCC.

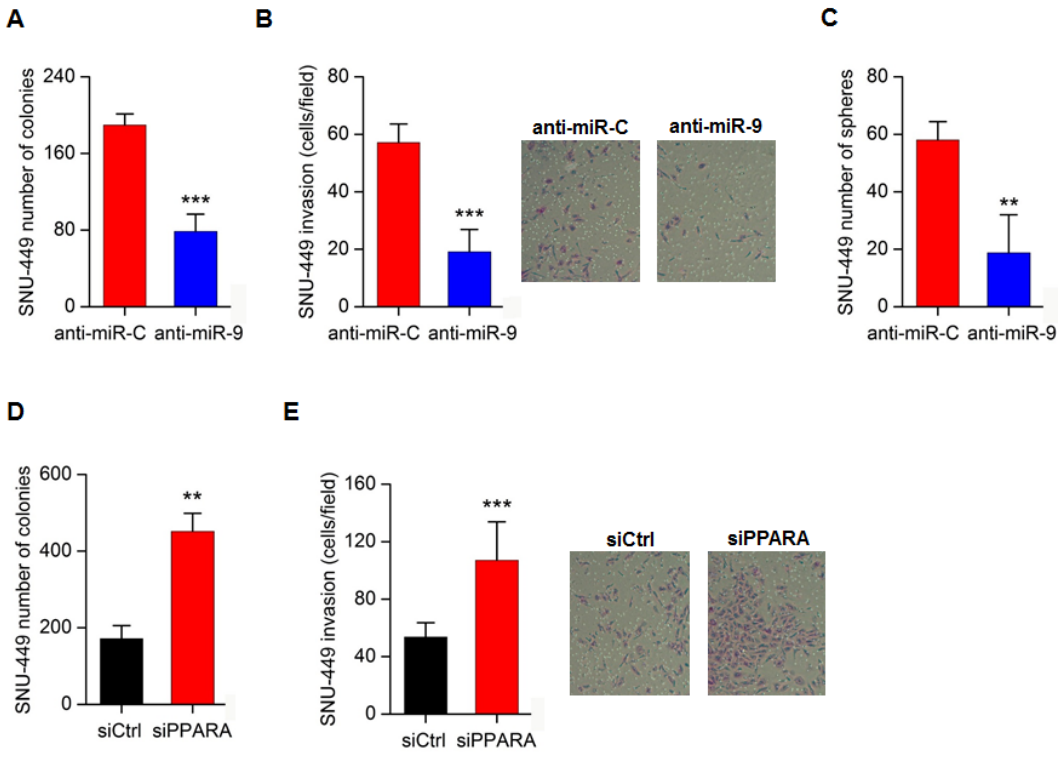

Figure 10. Effects of miR-9 inhibition on liver cancer cellular properties. (**A**) Soft-agar colony formation assay; (**B**) cellular invasion assay and (**C**) tumor sphere formation assay in SNU-449 cells transfected with an antisense microRNA negative control (anti-miR-C) or an

antisense microRNA-9 (anti-miR-9). (**D**) Effects of PPARA inhibition by an siRNA (siPPARA) or an siRNA negative control (siCtrl) on the ability of SNU-449 cells to form colonies in soft-agar and (**E**) SNU-449 cell invasiveness. All data are represented as mean ± SE. ***P<0.001, **P<0.01.

MiR-9/PPARA/CDH1 pathway expression levels in HCC tissues.

To study the human relevance of the miR-9/PPARA/CDH1 signaling pathway, we examined PPARA and CDH1 expression levels in 24 HCC and 11 control liver tissues. Real-time PCR analysis showed that CDH1 had >40% down-regulation of its mRNA levels in HCC relative to controls (**Figure 11A**) and PPARA had >50% reduced levels in HCC relative to control tissues (**Figure 11B**). Furthermore, we performed correlation analysis, evaluating the significance of correlation between miR-9 and PPARA or CDH1 mRNA levels in HCC tissues. Consistent with our *in vitro* findings, miR-9 was inversely correlated with both CDH1 (R^2=0.5824) (**Figure 11C**) and PPARA (R^2=0.7131) (**Figure 11D**) mRNA levels in HCC tissues. Taken together, these findings reveal the human relevance of the miR-9 signaling pathway in HCC oncogenesis.

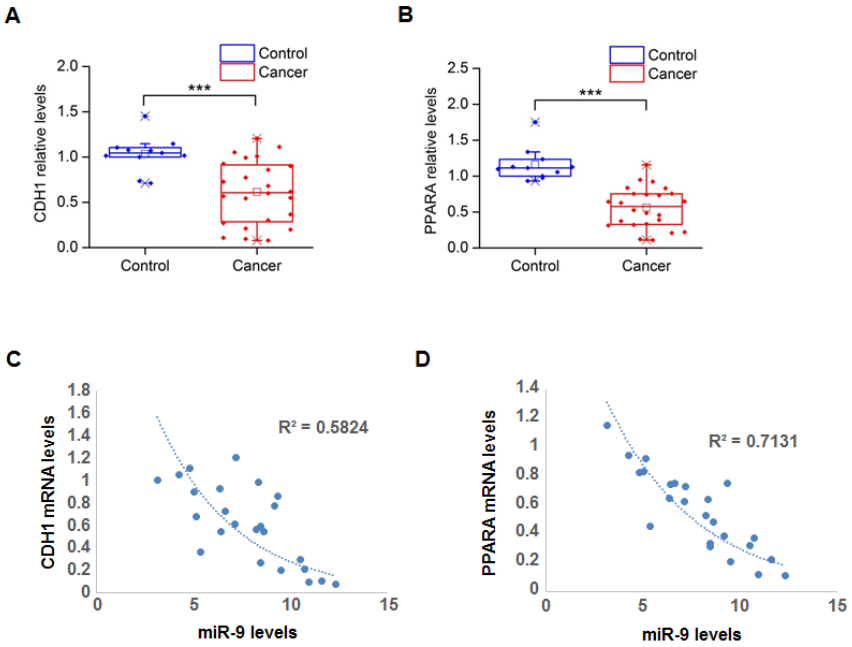

Figure 11. MiR-9 signaling pathway levels in HCC tissues. (**A**) CDH1 and (**B**) PPARA mRNA relative expression levels in 24 HCC tumors and 11 control liver tissues assessed by real-time RT-PCR analysis. Gene expression levels were normalized to the levels of GAPDH and β-actin. Normalized gene expression levels were quantified relative to the levels of a given control tissue. (**C**) Correlation analysis between miR-9 and CDH1 mRNA levels in 24 HCC tissues. (**D**) Correlation analysis between miR-9 and PPARA mRNA levels in 24 HCC tissues. Data are represented as mean ± SE. ***P<0.001, in comparison to control.

2.4 Discussion

Different signaling pathways have been implicated in HCC pathogenesis [175], however the role of non-coding RNAs has not been studied extensively until recently. Non-coding RNAs consist primarily of the microRNAs and long non-coding RNA (lincRNAs) and several studies have implicated their role in HCC initiation and progression [130,176-178]. Specific microRNA signatures have been identified to be deregulated in HCC patient tissues and also to correlate with different clinicopathological parameters [179,180]. Furthermore, microRNAs have been associated with hepatitis infection, cirrhosis and patient survival [141].

In this study, we have screened the human microRNAome, aiming to identify microRNAs that are potent regulators of HCC invasiveness. Interestingly, we found 28 microRNAs to affect significantly (>2-fold) the invasiveness of SNU-449 liver cancer cells. Five of these microRNAs behaved as HCC invasion inducers, while 23 microRNAs as HCC invasion suppressors. This screen revealed novel microRNAs potentially involved in HCC pathogenesis and also validated findings from previous studies. Specifically, microRNAs such as miR-21, miR-29a/b, miR-26a, miR-101, miR-122a, miR-124a, miR-375 and let-7a/b have been correlated with HCC

pathogenesis through regulation of essential signaling pathways [126,151,155,156,168,181-183]. More recently, we have identified that miR-24 is part of a feedback loop circuit involved in HCC pathogenesis [130]. On the other hand the role of miR-9, miR-148b, miR-203 and miR-507 in HCC pathobiology is not well understood. Recently, high miR-9 expression levels were found to be correlated with poor prognosis in HCC patients [184]. Furthermore, miR-148b expression was found to be decreased in HCC patients [185], however it is not known which signaling pathways are mediators of miR-148b activity in HCC. In addition, it has been shown that miR-203 is suppressed in HCC tissues due to DNA methylation on its regulatory area [150]. Finally, nothing is known regarding the role of miR-507 in HCC pathogenesis.

Here, we provide evidence that miR-9 affects different liver cancer cell properties, including liver tumor sphere formation. When liver cancer cells are placed in low attachment plates or in suspension, they have the ability to form liver tumor spheres, which potentially represent the cellular population harboring tumor-initiating properties [186,187]. Here, we evaluated for the first time the role of miR-9 to affect the growth of these liver tumor spheres and identified that miR-9 overexpression induced the formation of liver spheres derived from SNU-449 cells, suggesting its potential involvement in early stages during HCC oncogenesis. On the other hand, inhibition of miR-9 by an anti-sense microRNA-9 molecule, suppressed the growth of SNU-449-derived tumor spheres.

Bioinformatics and molecular analyses revealed that miR-9 is involved in HCC pathogenesis through direct regulation of CDH1 and PPARA genes, by binding on their 3'UTR regions. Previous studies have shown that reduced expression of CDH1 correlate with poor outcomes in HCC patients [188]. Consistent with our findings, Tan HX et al. showed that miR-9 was significantly up-regulated in primary HCC tumors with metastases in comparison with those without metastases [189]. In the same study, CDH1 levels were found to be up-regulated after miR-9 inhibition. Other studies have shown that high levels of CDH1 have been correlated with

suppression of liver carcinogenesis [190]. In addition, we found that miR-9 overexpression resulted in increased vimentin levels, which is a well-known mesenchymal marker correlated with CDH1 loss of expression in HCC [191]. More importantly, the role of PPARA in HCC pathogenesis has not been previously described. PPARA is a transcription factor that has been implicated in hepatic steatosis [192] and hepatic metabolic homeostasis through regulation of the hepatocyte nuclear factor-4 alpha (HNF4A) gene [193]. Interestingly, we have recently found that HNF4A is a tumor suppressor gene in HCC pathogenesis [130]. Furthermore, it has been described that there is a positive correlation between CDH1 and the PPARA signaling pathways [173,174]. Our analysis revealed that there is not only a positive correlation between PPARA and CDH1 mRNA levels in HCC, but also that PPARA regulates CDH1 mRNA expression levels in HCC. This observation is very interesting and novel, since miR-9 is using two discrete molecular pathways to suppress CDH1 expression in HCC. First, miR-9 directly suppresses CDH1 mRNA levels through binding on its 3'UTR and in the second indirect mechanism miR-9 suppresses PPARA mRNA levels directly, resulting in decreased CDH1 levels. Overall, these data suggest that microRNAs could use complementary mechanisms to regulate a specific downstream signaling target.

2.5 Future Directions of microRNA targeted therapies in HCC

MicroRNA deregulation could have therapeutic effects by suppressing the growth of cancer cells without affecting the growth of normal cells. These findings that have been identified through gain- and loss-of-function studies have enforced the development of microRNA-based therapeutics in the last few years, through restoration of the expression levels of down-regulated microRNAs or suppression of up-regulated microRNAs in cancer.

2.5.1 Restoration of tumor suppressor microRNAs in HCC

MicroRNAs whose levels are decreased in tumors and play role as tumor suppressors could potential be therapeutic targets. Thus, the development of microRNA mimics which could lead to restoration of microRNA expression represents an important treatment option for liver cancer patients. Two recent studies highlight the therapeutic potential of microRNA mimics in liver cancer. Specifically, it was revealed that restoration of miR-26a expression levels suppressed tumor growth in a liver cancer mouse model [153]. The systemic adeno-associated (AAV) viral delivery of miR-26a suppressed MYC-induced liver oncogenesis through suppression of cyclins D2 and E2, did not affect the growth of normal hepatocytes and was well tolerated from most tissues. In addition, we have demonstrated that restoration of the expression levels of miR-124 was very effective in suppressing chemical-induced liver carcinogenesis. In specific, systemic administration of miR-124 suppressed liver cancer growth in diethylnitrosamine (DEN)-treated mice through suppression of the IL6/STAT3 inflammatory pathway. Furthermore, miR-124 administration resulted in restoration of miR-124 physiological levels in the liver without having any side effects in major organs such as the kidneys, heart, pancreas and lungs, suggesting that miR-124 restoration could be a clinically viable therapeutic approach for liver cancer patients [130].

2.5.2 Suppression of oncogenic microRNAs in HCC

On the other hand microRNAs that are overexpressed and are considered the driver of oncogenesis are another potential target. There is a lot of effort to develop stable microRNA inhibitors that could eventually be used therapeutically in cancer patients. Furthermore, different chemical modifications in antisense-microRNAs have been developed aiming to enhance the specificity, potency and bio-availability of these inhibitors. Recently, it has been identified that

the locked nucleic acid (LNA) modification increases the stability of antisense microRNAs, which could be delivered by intra-tumoral, intraperitoneal and intravenous injections with minimal toxicity [91]. Upon systemic delivery, the vast majority of the LNA-microRNA is up-taken by the liver, suggesting the potential therapeutic efficacy and specificity of these molecules to suppress liver cancer growth [194]. Thus, it is important to develop LNA-antisense-microRNAs against major up-regulated microRNAs in liver cancer and evaluate their efficacy in cellular and animal models. Specifically, miR-21 has been identified to be one of the top up-regulated microRNAs in liver cancer patients; it regulates essential cancer signaling pathways and is a very attractive target for development of anti-miR-21 therapeutics [120]. In addition to the LNA technology, a recent study revealed that a 2'-O-methyl phosphorothioate-modified anti-miR-221 oligonucleotide suppressed efficiently liver cancer cell proliferation [195]. Furthermore, in the same study they have developed a cholesterol-modified isoform of miR-221 (chol-anti-miR-221) which exhibited improved pharmacokinetics and liver tissue distribution in comparison to the unmodified miR-221. Importantly, intravenous administration of chol-anti-miR-221 suppressed effectively liver tumor growth in vivo. These promising data suggest the potential use of chol-antimicroRNAs as therapeutics in liver cancer patients. Both the LNA- and cholesterol-modified microRNAs could be used to target highly oncogenic microRNAs in liver cancer. In addition to miR-21 and miR-221, recently we have identified that miR-24 overexpression is able to transform immortalized hepatocytes, while its inhibition suppressed liver cancer growth in xenograft tumors [130]. Also, inhibition of miR-30d has been identified to reduce the metastatic potential of liver tumors [136]. These findings propose that both miR-24 and miR-30d serve as appropriate targets for the development of chemically-modified inhibitors that could be further tested in different liver cancer animal models.

2.5.3 Delivery methodologies for microRNAs in HCC

Nevertheless, it is important to develop and use different approaches in order to deliver microRNAs in the target tissue with high degree of specificity and minimal side effects in major organs. MicroRNAs and antisense-microRNAs due to their nature they are concentrated and metabolized in the liver, suggesting that these molecules could achieve high specificity and effectiveness of delivery in liver cancer patients.

Below, we described different delivery methodologies that have been used successfully to transfer microRNAs or their inhibitors in the liver (**Figure 12**).

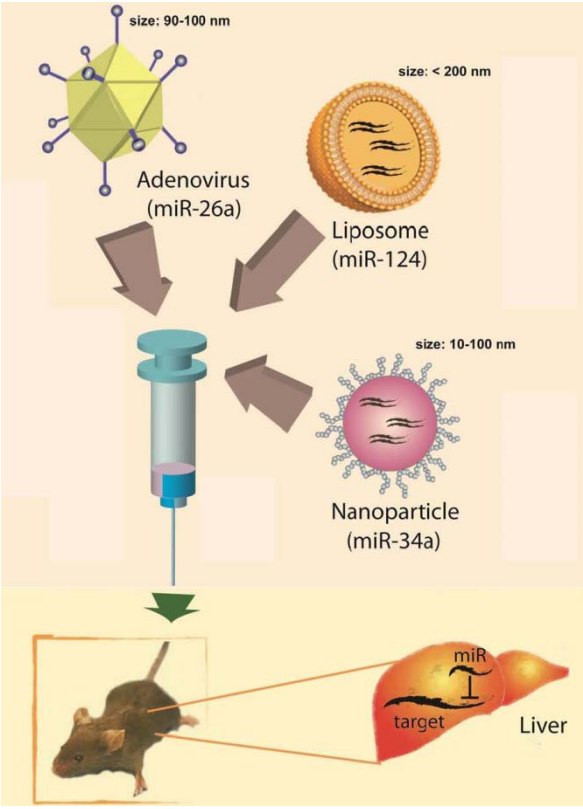

Figure 12. Delivery Methodologies for transferring microRNAs or their inhibitors in the liver. A) MicroRNA delivery in adenoviral vectors (AAV); B) Liposomal delivery of microRNAs and C) MicroRNAs encapsulated in nanoparticles.

1. Nanoparticle delivery of microRNAs in liver tumors

Recent studies have revealed that nanoparticles can be used to encapsulate microRNAs which could be delivered therapeutically to suppress tumor growth. Specifically, Pramanik et al. have developed a nanovector in order to transfer miR-34a in pancreatic tumor cells in vitro and in vivo [196]. This plasmid DNA-complexed nanovector is approximately 100nm in diameter and showed no histopathologic or biochemical evidence of toxicity upon intravenous injection. Furthermore, reconstitution of miR-34a expression suppressed tumor growth in xenografts. Due to the fact that miR-34a is highly down-regulated in liver cancer patients and is a component of the p53 transcriptional network, the miR-34a nanovector could be potentially used to evaluate its effectiveness and specificity on liver tumor growth suppression in vitro and in vivo.

2. Liposomal delivery of microRNAs in liver tumors

Liposomes have been extensively used for delivery of small RNA molecules (siRNAs or microRNAs) in different human tissues. Recently, we have identified that liposomal delivery of miR-124 suppressed liver tumor growth in different liver cancer mouse models. Specifically, a mix of liposomes and miR-124 as a single stranded RNA was administered intravenously in mice that have developed liver cancer. Due to the fact that high concentrations of the liposomal-microRNA could have side effects, we performed multiple experiments in order to identify the concentration of liposomal-miR-124 that would result in restoration of miR-124 expression in similar levels that is found in normal hepatocytes. That concentration was very effective to suppress liver tumor growth without any side effects or toxicities in major organs. Furthermore, miR-124 expression was found to be restored even 7 days post treatment, allowing administering miR-124 in a weekly basis [130]. This approach has the potential to be clinically viable and it seems

safer 24 than the use of viral vectors, which could induce immune responses and affect the expression levels of other host microRNAs.

3. Viral delivery of microRNAs in liver tumors

In a study described above, miR-26a has been identified to suppress tumor growth in a liver cancer mouse model [153]. The expression of miR-26a was restored by an AAV-based vector system which was very effective and did not affect the growth of normal hepatocytes. One advantage of this technology is that microRNAs delivered in AAV vectors are continuously transcribed, allowing sustained high expression levels in the target tissue. In comparison to retroviral delivery systems, AAV systems carry substantially diminished risk of insertional mutagenesis since viral genomes persist primarily as episomes [197]. In addition, the availability of multiple AAV serotypes allows efficient targeting of many tissues of interest [198,199]. All these data indicate that AAV delivery of microRNAs, that are down-regulated in liver cancer patients, could be a viable therapeutic approach that needs to be further tested experimentally in liver cancer animal models.

2.6 Conclusion

The realization that microRNAs play an essential role in initiation and progression of liver oncogenesis has provided a novel perspective on our understanding of pathophysiological mechanisms and has offered new therapeutic options for disease modification. The ability to modulate microRNA expression levels in the liver through delivery of microRNAs or microRNA inhibitors with minimal toxicity suggests that microRNAs could be clinically viable therapeutic approaches for liver cancer patients. The most advanced microRNA therapeutic approach developed to date is an LNA-miR-122 inhibitor as a treatment for hepatitis C virus (HCV) infection [200]. Importantly, delivery of the LNA-miR-122 inhibitor suppressed HCV replication in primates and currently is being tested in humans [201,202]. Taken together, microRNAs and microRNA inhibitors have a great potential to be used a therapeutic agents in liver cancer patients. However, extensive studies need to be performed in different liver cancer animal models and develop novel delivery technologies in order to optimize their effectiveness and minimize their side effects. Hopefully microRNA-9 inhibitor will be a future novel approach of managing hepatocellular carcinoma. Our ultimate goal is to bring this molecule to clinical practice. Upon completion of preclinical studies and with the financial support of the pharmaceutical companies who are making those novel drugs we hope to start the first investigator initiating phase one clinical trial for this fatal disease.

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