UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXII CICLO DEL DOTTORATO DI RICERCA IN

BIOMEDICINA MOLECULARE

TRANSLATIONAL STUDIES ON MICRORNA IN HEPATOCELLULAR CARCINOMA: FROM PREDICTIVE AND PROGNOSTIC BIOMARKERS TO MOLECULAR EFFECTORS

Settore scientifico-disciplinare: BIO/11

DOTTORANDO
MUHAMMAD YOGI PRATAMA

COORDINATORE
PROF. GERMANA MERONI
Universita degli studi di Trieste

SUPERVISORE DI TESI
PROF. CLAUDIO TIRIBELLI
Fondazione Italiana Fegato

CO-SUPERVISORE DI TESI
DOTT. DEVIS PASCUT
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Summary

Translational Studies on microRNA in Hepatocellular Carcinoma: From Predictive and Prognostic Biomarkers to Molecular Effectors

Muhammad Yogi Pratama

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, represents the seventh most frequent cancer and the fourth leading cause of cancer-related death worldwide. Due to unspecific sign and symptoms along with the inneficacy of current non-invasive diagnostic tools for detecting early stages HCC, majority of patients are diagnosed at advanced stages that is no longer eligible for curative treatments. Moreover, the long-term outcome of either curative and non-curative treatments remain unsatisfactory, which partly caused by intra and interheterogeneity of HCC and its complex molecular interplay. To overcome these problems, a better diagnostic and predictive biomarker might be one of the most feasible strategies. The characteristic of MiRNAs, that are proved to have essential roles in various cancer pathways and found to be stable in biological fluids, holds a potential value as non-invasive clinical biomarkers in HCC. The present study inclue three tasks whose aims are:

- **Task 1** - Identify circulating miRNAs as predictor of early HCC occurrence in high-risk population setting, specific in DAA treated chronic HCV patients.
- **Task 2** - Identify circulating miRNAs as a prognostic non-invasive biomarker after HCC treatments.
- **Task 3** - Identify putative targets of each potential miRNAs and their cellular involvement in HCC pathway by *in silico* target prediction methods and *in vitro* approaches using miRNA transfection methods in HCC cell model.

Result and discussion

Task 1: We performed a circulating miRNA profiling analysis of cirrhotic patients treated with DAA before and after therapy initiation, in order to identify miRNA biomarkers predicting the risk of HCC occurrence. Our group of patients consisted of 15 patients developing HCC after DAA treatment (HCC+) compared to 15 patients nor developing HCC (HCC-) within 12 month after SVR. Through microarray and qRT-PCR analysis, we confirmed differently expressed miR-3197 expression between HCC+ vs HCC− patients at T0 (before the initiation of DAA) ($p< 0.05$) with diagnostic performance of AUC values of 0.78 with a sensitivity of 79% and specificity of 70% in distinguishing both groups at T0. We also distinguish the expression of miR-3197 in all the cirrhotic patients from healthy individuals with a sensitivity and specificity of 76.5% and 53.5%, respectively, and HCC+ from healthy individuals with a sensitivity and specificity of 68.4% and 67.6%, respectively (AUC= 0.75 (95% CI 0.60-0.85), $p< 0.001$). Taken together, miR-3197 represent a promising tool for the identification of patients at risk such as HCV patients undergo DAA treatment.

Task 2: We performed a longitudinal study analyzing the expression of serum miRNAs in the cohort of 105 HCC patients before treatments (T0), one (T1) and six months (T6) after treatments. Patients were then separated based on several prognostic variables consist of therapy response (TR), disease-free survival (DFS), and overall survival (OS). High expression of miR-4454 ($p=0.02$) and miR-4530 ($p=0.04$), and low expression of miR-4443 ($P=0.05$) at T0 were significantly associated with complete response to curative treatments. The panel of the three miRNAs can distinguish complete responder (CR) from partial and non-responder (PR) with an AUC of 0.84, sensitivity and specificity of 72% and 75%, respectively. High expression of miR-4454 ($p=0.03$) and miR-4530 ($p=0.015$) were also associated with DFS > 6 months in patients receiving curative therapies with satisfactory potential to distinguish DFS with AUC of 0.81, sensitivity and specificity of 79% and 72%, respectively. For non-curative treatments (TACE), we observed the potential of miR-4492 distinguishing CR and PR ($p=0.01$) with a trend of upregulation in CR from T0 to T1 compared to PR ($p=0.03$). Mir-4492 was able to differentiate CR compared to PR of TACE with AUC=0.84 and sensitivity and specificity of 84.6% and 71%. For OS, we observed significantly different expression of miR-4507 ($p=0.00037$) and), and miR-3185 ($p=0.014$) to distinguish patients with shorter and longer OS. Higher Expression of miR-4507 and miR-3185 was significantly associated with longer OS with HR of 1.98 ($p=0.016$) and 2.02 ($p=0.0086$), respectively. Taken together, we identified a panel

of novel miRNAs that were never reported as non-invasive prognostic biomarker candidate in HCC. As we observed different miRNAs candidate for each prognostic parameter and types of treatment, we also underlined the the specificity on utilizing specific miRNAs as predictive biomarker for specific type of therapy. This result might be in line with the future goal to apply individualized treatment protocols to every single HCC patients.

Task 3: Through *in silico* prediction approach, we discovered several cancer-associated target genes from our miRNAs candidate from task 1 and task 2, and validate their expression along with our miRNAs panel (task 1 and 2) in ten paired tumoral and distal HCC tissues. MiR-4454 expression were downregulated in the tumoral tissues while conversely, the expression of BAG5, DLG5, and EIF4A2, were upregulated. However, from *in vitro* analysis, transfection of 50nM of miR-4454 in JHH6 cell line was able to corfirm the targeting of DLG5, showing a 36% decrease ($p=0.004$), there were no significant differences observed in the expression of EIAF4A2 and BAG5, thus indicating that, at RNA level, the putative target of miR-4454 is DLG5. Through a scratch-wound assay, cells transfected with miR-4454 mimics had a deceleration of the wound closure and migratory potential to 50% compared to control, validating the functional role of miR-4454 to represses the migration of tumor cells by targeting DLG5

Chapter 1

Introduction

1. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, accounting for 75-85% cases of liver malignancy. It represents the seventh most frequent cancer and the fourth leading cause of cancer-related death worldwide, contributing to 782.000 deaths in 2018 [1]. Currently, HCC remains as one of the most "difficult-to-treat" cancers due to the late diagnosis and its poor prognosis rate. Indeed, the vast majority of HCC are diagnosed at advanced stage that is no longer eligible for surgical approaches or liver transplantation [2]. In addition, the tumor heterogeneity plays an important role on this regard, causing the available therapeutic approach might not be suitable for all patients.

1.1. Epidemiology

The incidence of HCC varies between gender, as it represents the 7th most common tumor in males and the 13th in females, with a prevalence of 53/100.000 in males and 22/100.000 in females (male-to-female ratio= 2:1) [1]. The mean age at diagnosis varies according to the geographical distribution, patients are generally younger in Africa and China (between 55 to 59 years), 63-65 years in Northern America and Europe, while older in Japan with a mean age of 70-79 at time of diagnosis [3]. The highest incidence of HCC is documented in East asia, Sub-Saharan Africa, and Melanesia, where 85% of cases occur [1]. However, the incidence and mortality rates in western populations are estimated to be significantly increased in the decades,reaching more than 100.000 new case/year by 2030 [1,4]. The distribution of HCC incidences seems to be associated with the prevalence of the risk factors and with socio-demographic factors, suggesting a complex interplay between multiple genetic and environmental factors in the HCC occurence [5–7].

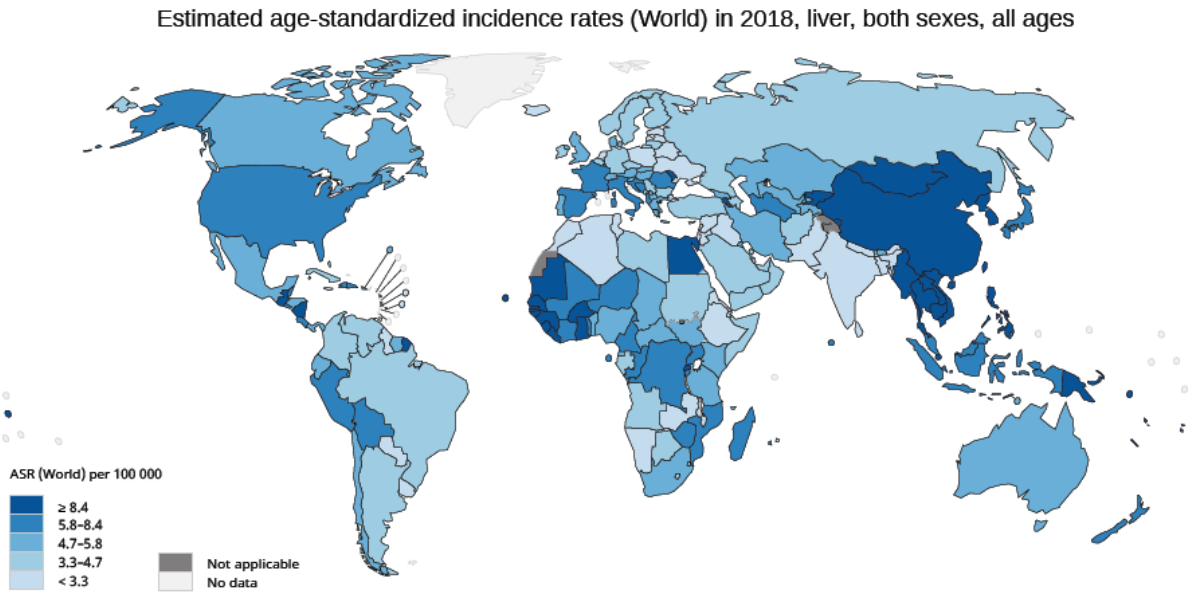


Figure 1. Worldwide distribution of HCC incidence in 2018, taken from Globocan, 2018, https://gco.iarc.fr/) [1]

1.2. Etiology

A multitude of etiological risk factors is shown to have a strong association with the development of HCC. Viruses such as hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV) give a strong contribution to tumorigenesis in the liver [8]. Thus, the incidence of HCC mirrors the geographical distribution of viral infection prevalence. In Sub-saharan Africa and Eastern Asia, the high-incidence rate is associated with high HBV prevalence, contributing to 50%-80% of all HCC cases in these regions [9]. Chronic HBV infection increases fifteen to twenty times the the risk for developing HCC with a mortality rate of 30-50%. HCV is the second most common risk factor for HCC in most of the western countries, accounting for 25% of the HCC cases worldwide [6,8,10]. The co-infection of viruses, HBC/HCV and HBV/HDV, is reported to increase the risk of developing HCC by two to six folds [11].

Other non-viral risk factors contribute to the HCC global incidence. The hepatic manifestation of metabolic syndrome, recognized as Non-alcoholic fatty liver disease (NAFLD), is closely associated with HCC [12]. Recent data shows an incidence of 10.6 per 1000 person-years of HCC among NAFLD patients with cirrhosis in the United States [13]. Alcohol abuse, especially with the concomitant chronic hepatotropic virus infection that increases the risk of developing HCC in this group of patients. An interesting study described that heavy

alcohol users (20-560g/week) with chronic HCV infection had 2.3 times higher chances of developing HCC. In fact, alcohol liver disease (ALD) is the main etiology of HCC in Europe [6]. In addition, obesity and diabetes mellitus (DM) are associated with an increased risk of HCC. Indeed, NAFLD patients with obesity or DM as comorbidities have twice higher probability of developing HCC compared to those with NAFLD alone [12]. Other factors can also be associated with the risk of HCC. Dietary exposure to aflatoxin B1 has been strongly linked to HCC in low-income countries due to the chronic exposure to aflatoxin-contaminated food products [14]. Androgen hormones are related to having a pro-oncogenic role in HCC [15]. Tobacco abuse also increases the risk of HCC even if not consistent data are available, even in the presence of HBV or HCV infections [16].

Regardless of the etiology, liver cirrhosis is considered the main pre-tumorigenic condition, being an "entrance point" for HCC [17]. Chronic inflammation-induced lesions in the liver, hepatic fat accumulation (steatohepatitis), and progressive fibrosis, may develop into cirrhosis in a spare of decades [17–19]. Studies estimate that 85% cases of HCC in chronic hepatitis B usually occur on a background of cirrhosis [20]. Moreover, in HCV infection, HCC rarely occurs in the absence of advanced hepatic fibrosis or cirrhosis, as it is estimated that 27% of cases of cirrhosis worldwide derive from chronic HCV infection [20–22]. Indeed the incidence of HCC is three times higher in patients with HCV-related cirrhosis, compared to ALD or NAFLD [23].

1.2.1. HCC Occurrence and Recurrence after the introduction of curative treatments for HCV

Despite the relevance of HCV as one of the main etiological factors, the incidence of newly diagnosed HCC derived from hepatitis C is expected to decrease after the introduction of new antiviral agents. Since 2013, the treatment of hepatitis C has dramatically improved after the presence of the new generation of direct-acting antiviral agents (DAAs) that have been extraordinarily effective, safe, and well-tolerated [24]. Indeed, these DAAs has shown a sustained virological response (SVR) rates exceeding 95% in real-life settings [24]. This represented a significant improvement in the treatment of hepatitis C with relevant implications parralel to the prevention of HCC. Indeed, the elimination of risk factor is expected to positively contribute to the reduction of HCC occurrence, especially in western countries. However, two recent studies from Italy and Spain revealed an unexpectedly high rate and risk of tumor occurrence and recurrence after HCV clearance [25,26]. There is an

ongoing debate about this aspect due to the conflicting shreds of evidence raised from several studies worldwide. Indeed, recent meta-analysis studies by Waziry *et al.* reported no evidence for differential HCC occurrence and recurrence in patients receiving either DAA or interferon therapy [27,28]. Considering the clinical relevance in the matter of this debate, scientists are now hypothesizing and collecting more evidences about the mechanisms, if any, possibly involved in tumorigenesis, even after the virus elimination. Despite the observations about the increased or stable occurrence or recurrence, what is important, is that the risk of developing HCC is not reduced after the DAA treatment, especially if the patients had a long history of persistent chronic viral hepatitis. It was hypothesized that the dramatic reduction of the viral load after DAA treatment has a great impact on the immune system of the patient [29]. In particular, the re-programming of immune system lead to a reduction of cancer immune surveillance that previously controlled the tumor development, whithin a certain extent. This mechanism involves the alteration of cytokine network that play a central role in cross-talk among immune cells and may contribute to cancer promotion and progression . One study has reported an increase of serum IL-6 in patients with recurrent HCC after DAA treatment which might be linked to the imbalance of immune system progressing to *de novo* HCC, even though more data are needed to support this evidence [25,30]. Other speculations about the increased occurrence or recurrence after initial response to DAA treatment might suggest the presence of new or already-developed oncogenic mechanisms already present within the underlying cirrhotic liver and responsible for the tumor growth [25].

1.3. Molecular Pathophysiology: Molecular Alteration in HCC

The development of HCC results from a complex multistep process that occurs from the interaction between genetic and non-genetic host factors such as environmental exposure and viral cause. Hepatocarcinogenesis largely depends from the presence of an underlying chronic liver disease and cirrhosis represents the most frequent precancerous condition present in 70-80% of HCCs **(Fig. 2).** Degeneration and necrosis of hepatocytes in cirrhotic liver will cause the replacement of liver parenchyma with fibrotic tissue and compromising the normal function of liver [31]. The formation of regenerative nodules constituted by proliferating hepatic cells and dysplastic foci, represents the initial step leading to the insurgence of HCC [32]. In the setting of cirrhosis, the sequential events originating HCC start from the development of pre-cancerous cirrhotic nodules with low-

grade dysplasia, called low-grade dysplastic nodules (LGDNs), to high-grade dysplastic nodules (HGDNs) that are strictly related to HCC **(Fig. 3)** [33,34]. Several histological and molecular elements associated with HCC develop during these phases, angiogenesis, for example, gradually increases during hepatocarcinogenesis from HGDNs to classic hypervascular HCC, in comparison with LGDNs. Other elements such as large and small cell change, higher nuclear/cytoplasmic ratio, nuclear atypia, thickened trabeculae, and reduced numbers of portal tracts are also observed in HGDNs and closely resemble early stages of HCC.

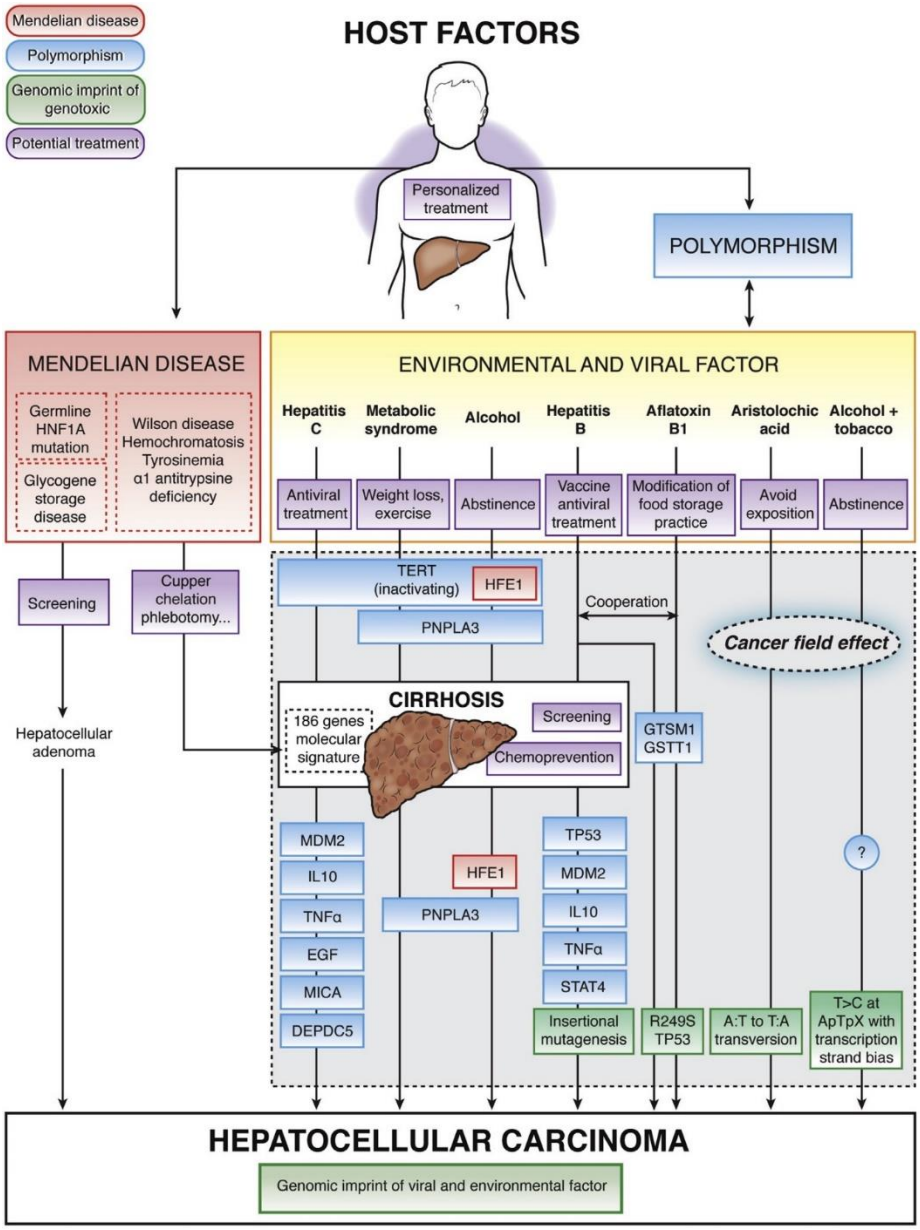


Figure 2. The compex interaction between genetic predisposision, environmental factors, and HCC occurrence. Mendelian disease (Red) and various enivironmental factors (Yellow)

influence a specific driver mutation (blue) in the progression to HCC. Genetic imprinting (green) also participates in HCC pathway. Several approaches (purple) can be done to prevent HCC development, taken from Zucman-Rossi *et al*. (2015) [33]

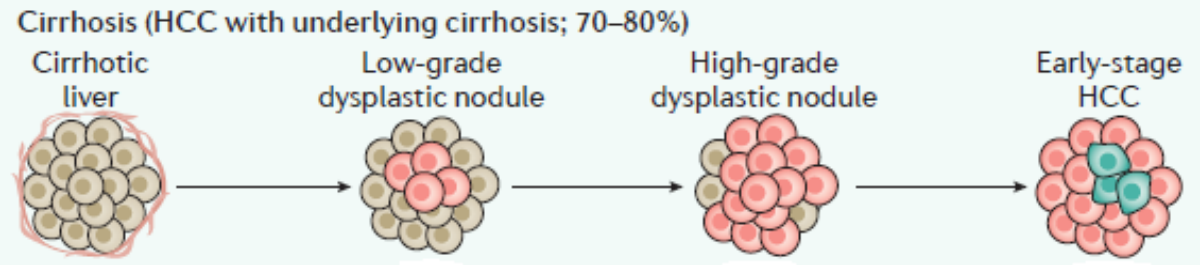


Figure 3. The development of HCC from liver cirrhosis. Cirrhotic liver will sequentially progress to low-grade dysplastic nodules (LGDNs), then to high-grade dysplastic nodules (HGDNs) containing more pre-cancerous cells, before originating early-stage HCC, taken from Llovet *et al.* 2016) [34]

Besides the morphological changes, a complex sequence of molecular alterations is continuously evolving in the spare of progression to HCC. Those alterations influence the tumor initiation, progression, metastasis and resistance to therapy by promoting various cancel hallmarks including proliferation, survival, invasion and/or immune evasion. Recent genomic sequencing identified 161 putative genetic alterations in HCC, in which 40-60% of somatic mutations are present in protein-coding genes [35]. Nevertheless, the majority of mutations are present in "passenger genes," involved in the indirect mechanism for cancer pathogenesis [36]. However, several gene mutations were identified as "driver genes," associated with a significant role of signaling pathways in HCC development [36]. As an example, three major mutations were associated with each of the etiological risk factors. *CTNNB1* with alcoholic liver diseases; *TP53* with HBV infection, while HCV infection mainly occurs without distinctive mutational patterns [33,36,37].

Telomerase (*TERT*) promoter mutations were recently identified as the most frequent somatic genomic defect in HCC, with an overall frequency of 60% to 90% [33]. *TERT*, a critical enzyme for replication of the chromosome termini in most eukaryotes, is a crucial component of the transformation process in many cancer cells [38]. The increased expression of *TERT*

leads to restoration of the anzymatic activity, which increase the proliferative capacity of neoplastic cells [39,40].

The WNT-β-catenin pathway, one of the main oncogenic pathways in HCC (up to 50% cases), is frequently activated via *CTNNB1* mutations, particularly in patients without viral hepatitis and well-differentiated tumors [41]. Indeed, previous studies have shown that 2.8-23.8 % patients with early-stage HCC have mutations in exon 3 of *CTNNB1* [42]. *CTNNB1* gene encoder for β-catenin that plays a crucial role in intercellular adhesion and communication, increased incidence of macro- and micro- vascular invasion, and had been associated with tumor size and multiplicity of nodules [41]. Another driver genes related to WNT-β-catenin pathway in HCC is *AXIN1*, approximately present in 5-19% of liver cancer specimens [41]. AXIN1 controls the level of β-catenin and serves as a negative regulator of this signaling pathway [41]. The loss of function mutations of *AXIN1* was associated with the proliferative class of HCC and related to the more aggressive phenotype [43]. However, conflicting evidence showed that *AXIN1* mutations could also develop without activation of WNT-β-catenin pathway, suggesting whether a mutational landscape of HCC should differentiate *CTNNB1* and *AXIN1* mutation from WNT-β-catenin group [43].

Cell cycle control is also the main molecular pathways involved in HCC [33]. *TP53* mutation, present in 13-48% cases of HCC, is one of the critical players in this pathway and strongly linked with HBx protein in HBV [33,38]. HBx binds to p53 and inactivates p53-dependent activities and deregulate cell-cycle check pointss blocking p-53 mediated apoptosis [44,45]. In general, the effect of mutant p53 expression has consequences in multiple pathological processes ranging from DNA damage repair, cell cycle arrest to apoptosis mechanism [46,47]. It negatively regulates the genes encoding for death receptors *CD95, TNF-R1*, and *TRAIL* [48]. Furthermore, it inhibits apoptosis in the outer membrane of mitochondria and regulates the expression of *VEGF-A*, strongly associated with angiogenesis [48,49]. Over the years, the role of p53 in HCC became even wider, since it was reported to be a key responder to inflammatory stress, and a protective anti-oxidative agent transcriptionally repressesing pro-oxidant genes such as nitric oxide synthases (*NOS*) 2s. *NO2* was also described to enhance the enzymatic activity of cyclooxygenase 2 (*COX2*), strongly associated with the repression of apoptotic pathway and the activation of WNT pathway that is known as a key contributor to liver carcinogenesis and poor survival in HCC patients [44,48]

Ooxidative stress and inflammation are constitutively activated in HCC due to disregulations in the nuclear factor erythroid 2-related factor 2 (NRF-2) or inactivation of Kelch-like ECH-associated protein 1 (KEAP1) in 5-15% of HCC cases [33]. In addition, the activation of neutrophils and Kupffer cells contributes to the enhancement of the oxidative status via cytokine production [50]. In HBV and HCV, viral particles stimulate the production of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, CXCL-8, and tumor necrosis factor (TNF) [50,51]. This leads to a persistent inflammatory signal in Kuppfer cells that up-regulate the immunoregulatory molecule of PD-L1, and stimulate the release of cytotoxic molecules such as granzyme B, perforin, and ROS, causing more severe stage of inflammation and fibrosis, the ideal setting for HCC development [51–53].

RAS-RAF-MAPK (MAP kinase) and the phosphoinositide 3-kinase (P13K)-AKT-mTOR pathways are frequently activated in HCC [33]. The RAS/RAF/MAPK pathway was found alterated in the majority of HCC at advance stage as a result of increased signaling from upstream growth factors and/or inactivation of tumor suppressor genes. *MAPK/ERK* is activated and upregulated in 58% of HCC cases [54,55]. However, the cellular mechanism behind its activation is not yet fully elucidated. Usually, the MAPK pathways regulate cell functions throught a complex interaction network involving other MAPK as well as different signaling pathways such as the p13k/Akt/mTOR, TGFβ/Smads, or WNT/β-catenin pathways [56,57]. The Ras/MAPK pathway plays a fundamental role in the control of cell survival and proliferation; thus, dysregulation is implicated in various cancer including HCC [58,59]. *RAS* mutations are frequently found in HCC experimental model, but rarely in HCC patients [60]. Meanwhile, down-regulation of several RAS/MAPK pathway inhibitors such as GAPs, RASSF proteins, and DUSP1 are implicated in human HCC [61]. Furthermore, the P13K/AKT/mTOR pathway was found in abberantly activated in 30-50% HCC cases [33,62]. This pathway antoganizes the MAP kinases pathway, as one pathway is activated when usually the other is inhibited, resulting in cancer cell survival and proliferation [62]. In HCC, the activation of mTOR pathway is associated with less differentiated tumors, poor prognosis, and early recurrence, as well as connected to EGF and PTEN pathways that accentuate the progression of HCC [63,64]. Current genomic studies have identified fifty driver genes linked to either mTOR, AMPK, or EGFR pathways [64].

Epigenetic regulators are also frequently altered in HCC. Epigenetic regulation is orchestrated by three major components, DNA methylation, histone modifications, and

chromatin remodeling [33,65]. Promoter hypermethylation plays a significant role in cancer through transcriptional silencing of tumor suppressor genes [66]. DNA methylation inhibitors such as azacitidine and decitabine can induce functional re-expression of abberantly silenced genes in cancer, causing growth arrest and apoptosis in tumor cells [67]. Multiple histones modifying enzymes, such as helicase-lymphoid-specific (HELLS), a chromatin remodeling enzyme, is remarkably overexpressed in HCC, and it correlates with aggressive features and poor prognosis in comparison to those with lower HELLS expression [68]. Law *et al.* described that overexpression of HELLS increased HCC cell proliferation and migration, while in contrast, depletion of HELLS led to a reduction of HCC growth and metastatic rate [68]. Meanwhile, AT-rich interactive domain 2 (*ARID2*) has been recently explored in the epigenetic field of HCC progression [69]. It was demonstrated that the suppression of *ARID2* accelerated G1/S transition and upregulation of cyclin D1, E1, and CDK4, accelerating tumor cell growth [69].

The results from the documented molecular alterations in HCC is one complex and heterogeneous interlooping event that might contribute to poor prognosis and survival. The presence of some driver genes raised the possibility to identify classification models including such molecular aleration for a better stratification of the patients in order to reach the best possible therapeutic approach.

1.3.1 Cancer Heterogeneity

HCC is considered a peculiar type of cancer due to the high heterogeneity of the disease that displays a variety of growth patterns, cytological features and different driver gene mutations. This heterogeneity negatively impact on the formulation of classification models, predictive algorithms and the treatment guidelines. It is clear that genetic predisposition on HCC is strongly influenced by etiological cause, as different mutation are found to be related with a specific etiological risk. Taken for example, TERT promoter mutation which are commonly found in HCC derived from Hepatitis C and metabolic syndrome, while Hepatitis B itself is associated with TP53 mutation [33,70]. Thus, it marks the wide-range of heterogenous tumor at the genomic and phenotypic level that varied between patients. In the other hand, different cellular sub clones would emerge during tumor growth as a result of selective pressure from microenvironment, carcinogenic exposure, or simply from a random acquisition of novel mutations [37]. It is believed that understanding and

identifying the molecular signatures in diverse heterogeneous subclasses of tumor might help in the prediction of treatment response to specific molecular therapies [37].

Genomic profiling of HCC has identified heterogenous molecular aberration shared by subgroup of tumors. Subsequent transcriptome profiles allowed the characterization and classification of HCC subtypes according to tumor aggressiveness [37,71]. For example, aggressive tumors are characterized by TP53 inactivation associated to alterations to its pro-oncogenic signaling pathways involving Myc and Akt. Those tumors are further subclassified according to expression of stemness marker genes such as *EPCAM* and late TGF-β [37,71]. Less aggressive tumors are characterized by *CTNNB1* mutations accompanied by overexpression of liver-specific WNT targets [70]. All the information related to genomic profiling of HCC are summarized in the work of Schulze *et al.* 2016. that provides new insight ino the complex molecular pathogenesis of HCC **(Table 1)** [37].

Lin et al. 2017. also analyzed an inter-tumoral heterogeneity from different HCC nodules in the same liver [72]. It is clear that tumor originating from *de novo* clones did not share the same genetic alterations but similar aberration methylation profiles, thus suggesting that common epigenetic alteration is already existed in the early pre-neoplastic lesion [72].

Molecular heterogeneity in HCC includes a consensus definition of crucial concepts that tumor arise from a single cell and trunk alterations at the early stage are the first pro-oncogenic molecular events arising during tumor evolution. Therefore, they would be clonally dominant and present in all tumor cells, even at the cases of multinodular HCC [73]. Some Early oncogonic mutation such as *TERT* promoter mutations and broad copy-number aberrations in chromosomes 1 and 8 were identified in 10.5% and 7% of displasic nudules, respectivelly, being the first events occurring in hepatocarcinogenesis. Overall, *TERT*, *TP53*, and *CTNNB1* mutations can be said as the most frequent trunk events in small HCC (sHCC) [73]. A recent study also identified a considerable heterogeneity in different tumor nodules from the same individual, at least in DNA sequence level [74]. It is suggested that the subclonal heterogeneity of cancer cells might be derived also after therapy, and progressed as a recurrence event [37]. Indeed, these subclonal mutations, evolving upon treatment exposure, might partake to the resistance towards HCC treatment [75].

Year	Author	Sequencing approach	Number of samples	Etiology	Cirrhosis	Candidate driver	De-regulated pathways
2012	Fujimoto *et al.*	Whole genome + Validation set	27 + 120	HCV (52%) HBV (41%)	52%	*TP53* (52%) *ATM* (19%) *IGSF10* (15%) *CTNNB1* (11%) *ARID1A* (11%)	
2012	Guichard *et al.*	Whole exome + Validation set	24 + 125	Alcohol (37%) HBV (24%) HCV (19%) NASH (5%)	38%	*CTNNB1* (33%) *TP53* (21%) *ARID1A* (17%) *AXIN1* (15%) *RPS6KA3* (10%) *CDKN2A* (7%)	Wnt signaling (49%) p53 signaling (33%) Chromatin remodeling (23%) PI3K/Ras signaling (13%) Oxidative stress (6%)
2012	Huang *et al.*	Whole exome + Validation set	10 + 100	HBV (100%)	no data	*TP53* (27%) *ARID1A* (13%) *SAMD9L* (6%) *ARID2* (4%)	
2012	Sung *et al.**	Whole genome	88	HBV (92%)	66%	*TERT* (20%) *KMT2B* (10%) *CCNE1* (5%) *SENP5* (3%) *ROCK1* (2%)	
2013	Cleary *et al.*	Whole exome	87	HBV (43%) HCV (21%) Alcohol (11%)	56%	*CTNNB1* (23%) *TP53* (20%) *CPA2* (9%) *IGSF3* (9%) *KEAP1* (8%)	
2013	Kan *et al.*	Whole genome	88	HBV (92%)	63%	*TP53* (35%) *CTNNB1* (16%) *LRP1B* (11%) *JAK1* (9%) *AXIN1* (5%)	Wnt signaling (63%) JAK/STAT signaling (46%) Apoptosis (46%) p53 signaling (43%)
2014	Ahn *et al.*	Whole exome	231	HBV (72%) HCV (10%)	49%	*TP53* (32%) *CTNNB1* (23%) *RB1* (8%) *AXIN1* (7%) *SELPLG* (5%) *FGF19* (5%)	p53 signaling (37%) Wnt signaling (37%) Chromatin remodeling (34%) Cell cycle (22%) PI3K/Ras signaling (12%)
2014	Totoki *et al.*	Whole genome + Whole exome	608	HCV (42%) HBV (23%)	no data	*TERT* (54%) *CTNNB1* (31%) *TP53* (31%) *ARID1A* (8%) *AXIN1* (6%) *TSC2* (5%)	p53 signaling (72%) Telomere maintenance (68%) Chromatin remodeling (67%) Wnt signaling (66%) PI3k/mTOR signaling (45%) Oxidative stress (19%)
2015	Schulze *et al.*	Whole exome	235	Alcohol (41%) HCV (26%) NASH (18%) HBV (14%)	47%	*TERT* (60%) *CTNNB1* (37%) *TP53* (24%) *ARID1A* (13%) *ALB* (13%) *AXIN1* (11%) *CDKN2A* (9%)	Telomere maintenance (60%) Wnt signaling (54%) PI3k/mTOR signaling (51%) p53 signaling (49%) MAP kinase signaling (43%) Hepatic differentiation (34%) Epigenetic regulation (32%) Chromatin remodeling (28%)

Table 1. List of candidate driver genes mutation in HCC discovered by NGS analyses. the distinctive mutations were linked to etiology underlying the complexity and heterogeneity of hepatocarcinogenesis, taken from Schulze *et al.* (2016) [37]

1.4. Clinical Manifestation

HCC grows silently, making the diagnosis challenging before the development of later stages of the disease, especially in countries where sourveillance programs do not exist [76]. Generally, various clinical presentations are related to the extent of hepatic reserve at the time of diagnosis. In 90-95% of HCC patients, the triad of right upper abdominal quadrant pain, palpable mass, and weight loss are present [77]. The presence of cirrhosis give non-specific signs and symptoms of hepatic decompensation, such as jaundice, hepatic

encephalopathy and anasarca edema [76]. Malignant invasion of HCC to portal structure give perpetual symptoms of ascites, variceal bleeding or hematemesis [76]. Some symptomatic cases tend to present with unspecific symptoms such as abdominal pain, distension, and anorexia [78]. A fatal complication of HCC is tumour rupture, with patient experiencing hypotension, peritoneal irritation and severe abdominal pain [79]. Paraneoplastic symptoms manifested in HCC is bone pain associated with hypercalcaemia, that might be caused by osteolytic metastasis; other systemic symptoms include erhtyrocytosis, hypoglycemia and androgen insensitivity syndrome [77].

1.5. Diagnosis

Unlike for most solid cancer, imaging techniques represent the main available tool for HCC diagnosis [80]. A surveillance program for high-risk population relies on abdominal ultrasound (US) due to the absence of risks, non-invasiveness and moderate cost [81]. However, recent meta-analysis showed that ultrasound alone has only 47% sensitivity for the detection of early stage HCC [82]. Cross-sectional imaging approaches such as four phasic CT or dynamic MRI remain as essential non-invasive modalities for early stages of HCC [80,83]. However, when comparing the diagnostic performance of multiphasic CT, MRI and liver US the sensitivity of both CT and MRI reached only 53% and 62%, respectively [84]. Another study reports a pooled sensitivity of 82% and 66% for MRI and CT, respectively, suggesting superiority of MRI to find a true-positive confirmation for diagnosing HCC [85].

The 2018 European Association of the Study of the Liver (EASL) guidelines for HCC strongly recommends a dynamic contrast-enchanced MRI to identify the typical hallmarks of HCC **(Fig. 4)** [83], and they suggest a consistency in the results of at least two imaging techniques for the final diagnosis of HCC. Indeed, EASL guideline also referencing the American Association for the Study of Liver Diseases (AASLD) 2018 guideline for HCC **(Fig. 5)** strongly recommends either multiphase CT or MRI for initial diagnostic testing. In the latter guideline, imaging data are included into the CT/MRI Liver Imaging Reporting And Data System (CT/MRI LI-RADS) algorithm that assigns the observed lesion to its probability of being benign, HCC, or other hepatic malignant neoplasm such as cholangiocarcinoma; basing on the morphology, size and the characteristic of the surrounding liver environment [80]. However, regardless of which imaging modalities are utilized, the radiological hallmarks of HCC only present in a minority of patients with small tumours (<2cm), delaying a definitive diagnosis of

a suspicious lesion until exceeding 2cm, leading to increased treatment failures or recurrence [83]. As another drawback, both contrast-enhanced CT and MRI are rather expensive, and exposing patients to adiations with an increased risk of tumor development [86]. Therefore, it is now crucial to provide a reliable, safe and cost-effective diagnostic tools for a small lesion[83].

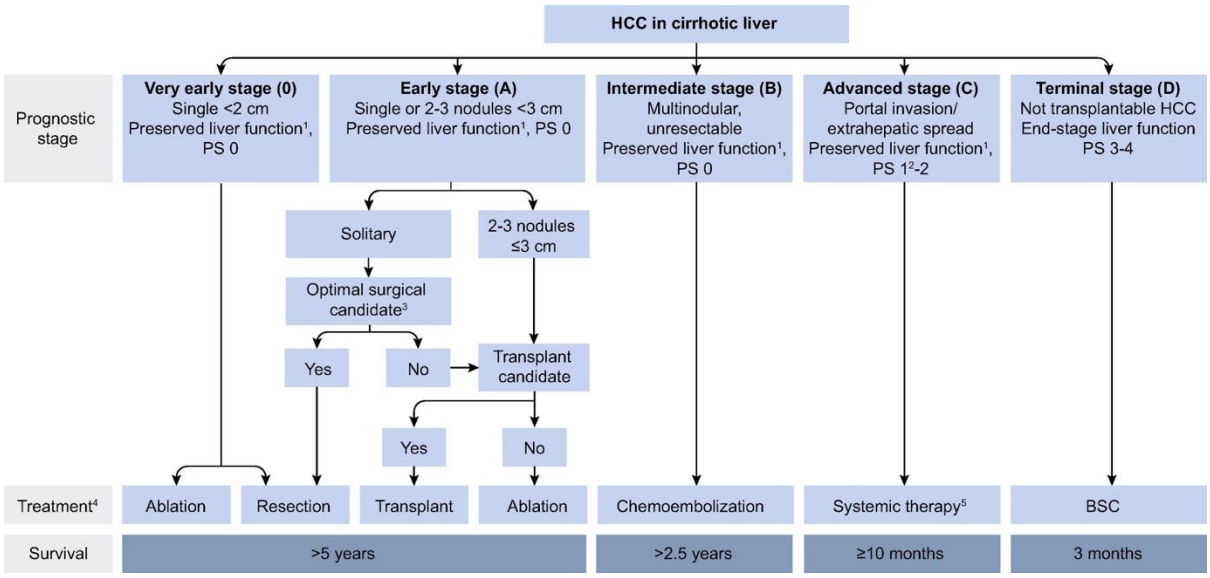


Figure 4. European Association for the Study of the Liver 2018 guideline for HCC diagnosis [87]

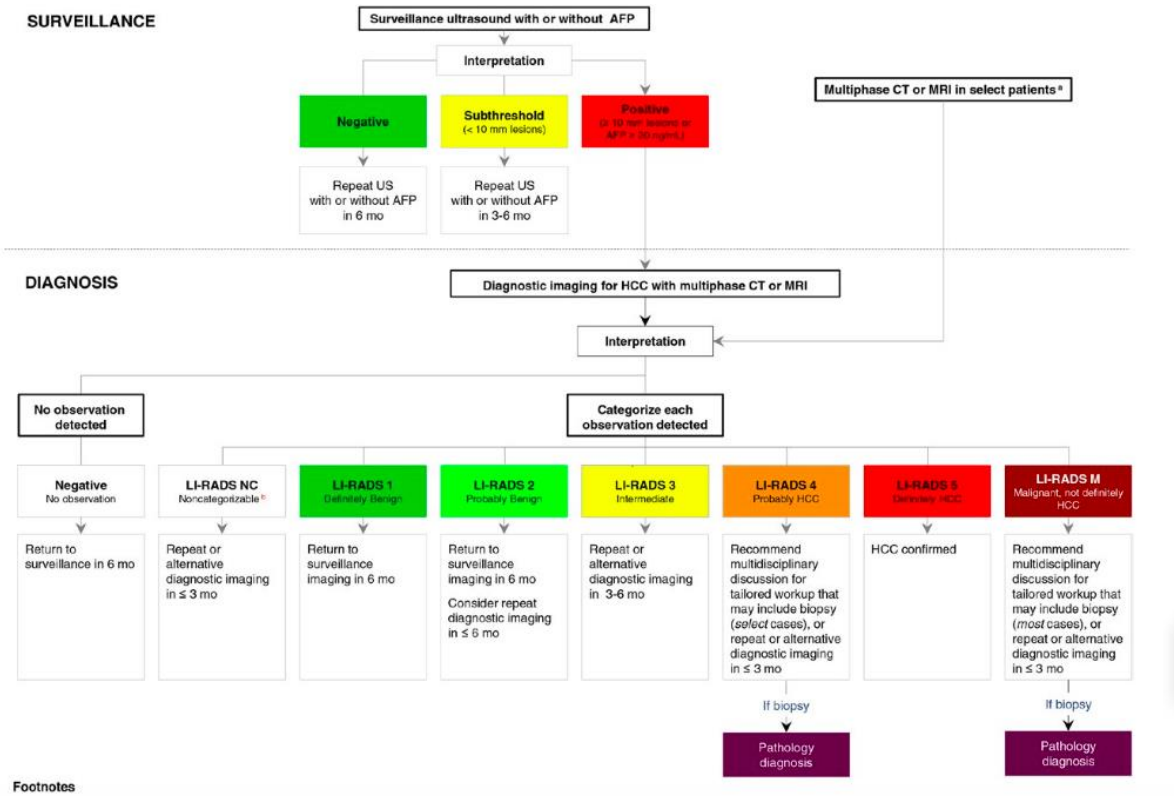


Figure 5. American Association for the Study of Liver Diseases (AASLD) 2018 guideline for HCC diagnosis [80]

Biomarkers from blood and other body fluids quickly become a major interest in the screening and diagnosis of HCC due to its non-invasiveness and straightforward nature. Alpha fetoprotein (AFP) that over the years became a widely used and broadly known biomarker for HCC, has been dropped from the current surveillance guidelines in Europe and United States due to the low sensitivity and specificity [80,83,86]. Almost 80% of small HCCs do not show increased levels of AFP especially tumors smaller than 3cm, where the sensitivity of AFP decreases to 25% [86]. Serum des-gamma-carboxy-prothrombin (DCP) also failed to obtain an optimal diagnostic performance, showing sensitivity values even lower than US [88]. Due to the lack of a reliable blood biomarker for HCC, over the past few years, research towards the development of novel HCC biomarker assays have dramatically increased. Cancer-specific DNA mutation, methylated DNA regions, long and short non-coding RNAs, proteins, and metabolites became eligible candidates as biomarker for multiple purposes in liver oncology.

1.6. HCC Staging

The prognosis and treatment of HCC depend on the tumor burden, patients' underlying liver disease, and liver function, which affect the eligibility for the treatment and the survival rate [89]. However, due to the variety of etiological background and extreme heterogeneity of HCC, developing a robust staging system and/or identifying prognostic marker for HCC are both challenging and urgently required [89].

Although there is no universally accepted staging system, the Barcelona Clinic Liver Cancer (BCLC) classification offers the most comprehensive tool for tumor classification and management since it includes an assessment of tumor burden, liver function, patients physical status, and cancer-related symptoms **(Fig. 6)** [80]. In a certain extent, the BCLC helps clinicians in stratifying patients according to the survival for each of the classes 0, A, B, C, and D [90,91]. A BCLC stage A (defined as an early-stage disease) includes patients with a Child-Pugh A or B status, diagnosed with one nodule of any size or a maximum of three nodules measuring < 3 cm [92]. A BCLC stage B (defined as intermediate-stage disease) corresponds to patients with a Child- Pugh grade A or B status, diagnosed with multiple nodules without vascular invasion or extrahepatic metastasis [92]. Patients with a Child- Pugh grade of A or B, vascular invasion or extrahepatic metastasis, and cancer-related symptoms (PS 1-2) are classified as BCLC C (defined as advanced-stage disease) [92]. Finally, patients with a Child-Pugh grade of C in any tumor stage and cancer-related symptoms (PS > 2) are classified as BCLC D (defined as terminal stage disease) [92].

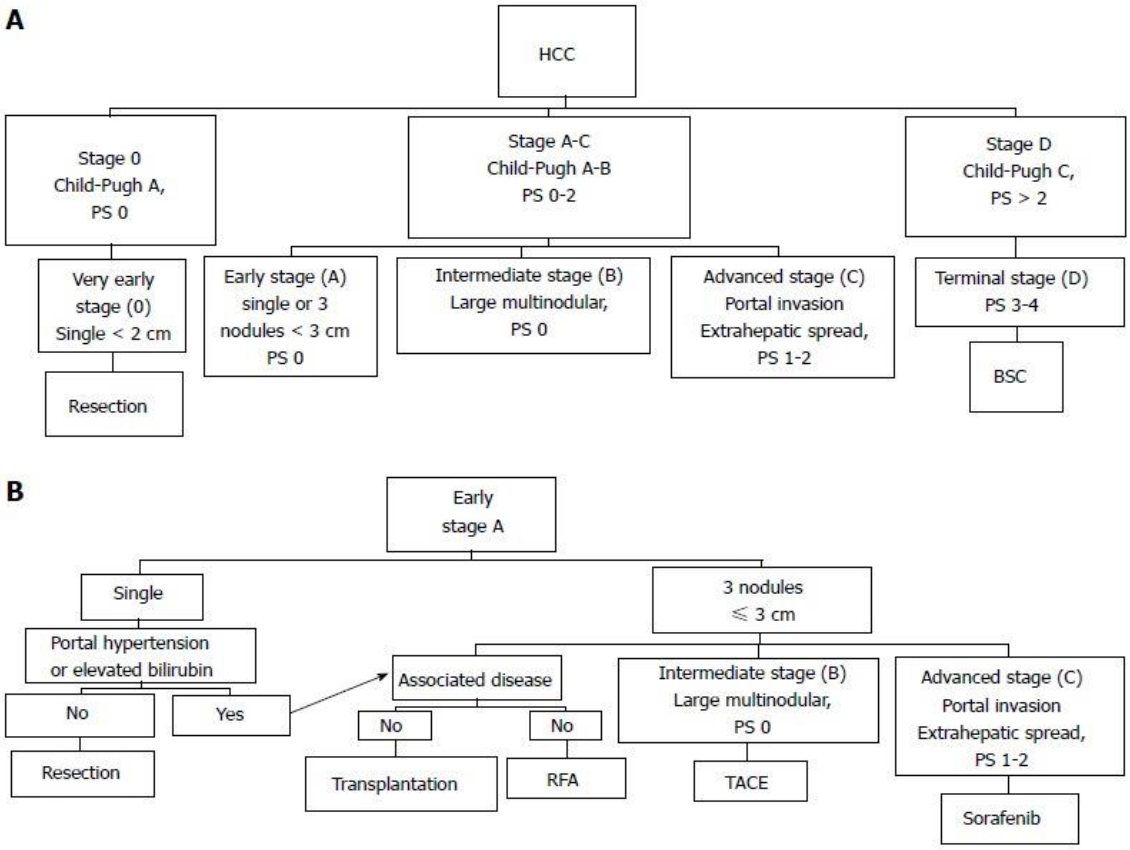


Figure 6. Barcelona Clinic Liver Cancer (BCLC) classification, taken from Llovet, 2014 [90]

1.6.1. Molecular Classification

Despite its well designed structure and its contribution to improve the management of HCC patients, there are limitations to the BCLC staging and treatment system. It is evident that a clear heterogeneity in the clinical manifestation and outcomes of the disease existed whithin each class. The precise understanding and translation of the morphologically heterogenous features of HCC, at the pathological level, as well as differential genetic features that influence the biology of HCC, might be a promising strategy to improve the classification, further to develop future targeted therapies and personalized treatments. On this regard, *Boyault et al*. (2007) investigated the transcriptome-genotype-phenotype correlations in HCC patients, identifying six subgroups of HCC, termed as G1 to G6. The classification takes into consideration clinical features, genetic alterations, gene mutations, promoter methylation of CDH1 and CDKN2A and HBV DNA copy number for each tumor [93]. Over the years, this classification was further expanded in large series of HCC with the implementation of a transcriptomic profile, immunohistochemistry marker expression and genetic alteration of the most recent gene involved in the disease **(Fig. 7)** [20,33].

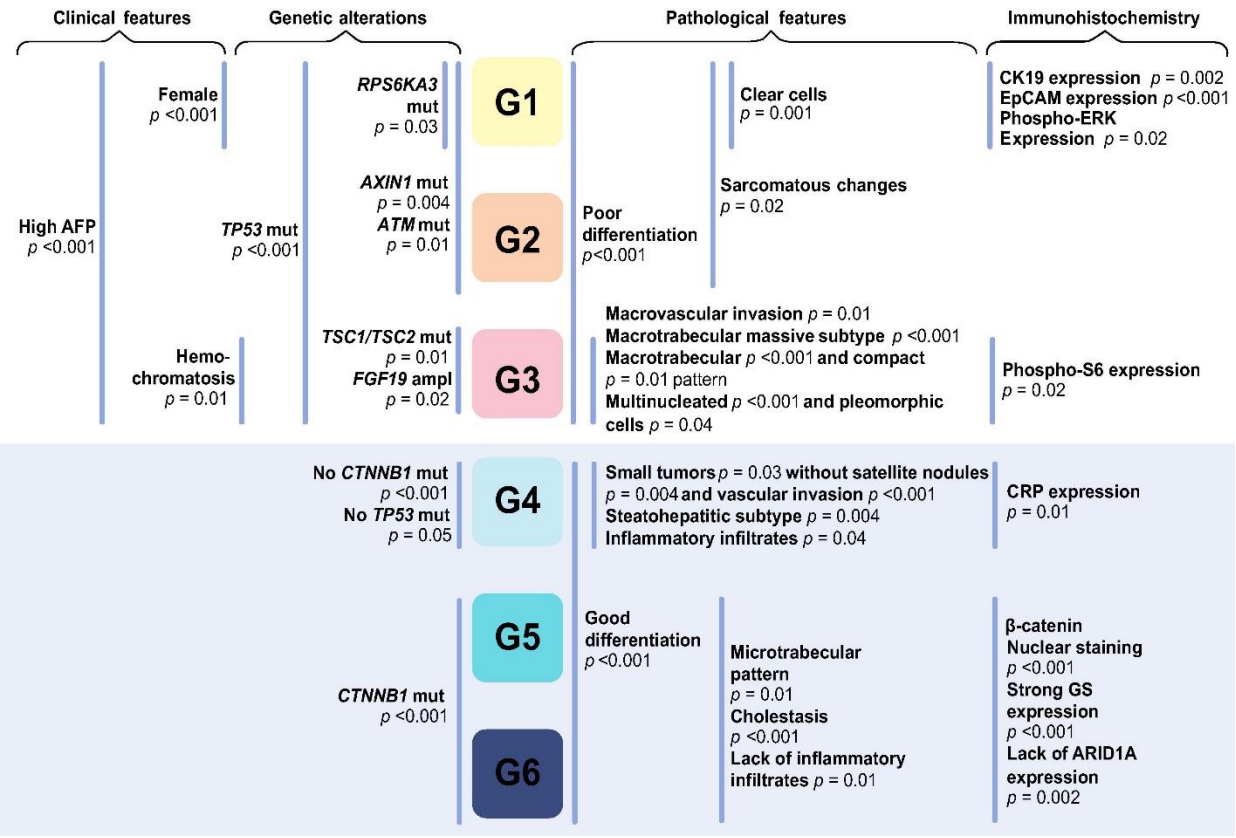


Figure 7. Molecular Classification of HCC. The six robust subgroups found and termed as G1-G6 according to their significant relationship with clinical, genetic, pathological features, and immunohistochemistry features. G1-G3 are considered as poor differentiation HCC while G4-G6 are considered as good differentiation HCC. Taken from Zucman-Rossi *et al*, 2010 [20]

Based on this classification, tumours belonging to the transcriptomic G1,G2,G3 subsclasses, are characterized by high cell proliferation and chromosomal instability, and several clinic-biological features: high AFP serum levels, female gender, and haemochromatosis. The mutation of *TP53* present in principally present in G1,G2,G3, while other mutations appear in some of subclasses; *RPS6KA3* (G1), *AXIN 1* (G1,G2), *ATM* (G1,G2), *FGF19,* and *TSC1/TSC2* alterations (G3). Histologically, G1,G2,G3 HCC are poorly differentiated with frequent macrovascular invasion, sarcomatous-like phenotipe, and macro-trabecular histological patterns. The G1 subtype showed a progenitor phenotype with both CK19 and EpCAM immunohistochemical expression. Activation of MAPKinase and P13K/AKT pathways are associated withG1 and G3 subclasses [94].

G4,G5,G6 tumor subclasses are characterized by low cell proliferation and chromosomal stability, and often well-differentiated. G4 HCC is additionally associated with

small tumor size, lack of satellite nodules, microvascular invasion, steatothepatitis subtypes and inflammatory infiltrates. G5,G6 subclasses are strongly related to *CTNNB1*-activating mutations, and further to microtrabecular pattern of growth, tumour cholestasis, and lack of inflammatory infiltrates. Immunohistochemical staining showed Wnt/beta-catenin pathway activation and lack of *ARID1A* expression [94]. The relevant information coming from this new suggested molecular classification is the strong relationship between molecular and pathological features with *CTNNB1* and *TP53* alterations occuring in the 57% all HCC [94]. In addition, the importance of the of this classification should be considered to envision the evolution of the actual treatments toward combined molecular-targeted therapies and personalized treatments based on the distinctive profile for each patient [95].

1.7. Treatment

Curative treatments for HCC include orthotopic liver transplantation (OLT), surgical resection and thermal ablation. Liver transplantation might be a definitive approach for eligible patients [96]. However, post-transplantation HCC occur in 10-20% of the patients [97]. Based on the BCLC grading system, resection is reserved for stage 0 (level of evidence 1) or A (level of evidence 2) [80,92]. Unfortunately, since the liver cancer usually occurs on the background of cirrhosis, the post-resection environment is still susceptible to recurrence [96]. Radiofrequency ablation (RFA) has recently become a potentially curative and minimally invasive approach but limited to HCC of 3 cm or smaller in Child-Pugh class A or B cirrhosis [98]. Concerning overall survival, some non-randomized comparative studies reported a similar outcome between RFA and resection, but with lesser adverse events [98–100]. Despite the presence of such options, the 80-90% of newly diagnosed HCC are not considered eligible for any curative treatments.

Non-curative therapies, which attempt to prolong survival by slowering tumor progression, include transarterial chemoembolization (TACE), transarterial radioembolization (TARE), stereotactic body radiation therapy (TARE), and systemic chemotherapy [87]. TACE remains as the recommended first-line therapy for BCLC B grade [87], improving the survival of patients with unresectable HCC [101]. However, the long-term outcome remain poor, and TACE can be associated with several contraindications, especially considering the high heterogeneity of HCC [102].

Molecular-targeted therapies for HCC has been an appealing option since the development of the multikinase inhibitor sorafenib, firstly described in the Sorafenib HCC Assessment Randomized Protocol (SHARP) trial in 2007 in a cohort of advanced HCC [103–105]. Sorafenib is an inhibitor of Raf-kinase and several tyrosine kinases receptors implicated in tumorigenesis, tumor progression, and vascularization. In most tumor types, Sorafenib induces apoptosis by down-regulating the anti-apoptotic protein Mcl-1 through a MEK/ERK-independent mechanism [106,107]. Sorafenib is also described to target pro-angiogenic and pro-fibrotic players such as receptor tyrosine kinase (c-Kit), Fms-like tyrosine kinase (FLT-3), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR-β) and other tyrosine kinases [108,109]. Unfortunately, even though Sorafenib was an achievement for HCC therapy, the outcomes are still far from being satisfactory [110]. Multiple phases 3 trials have failed to demonstrate outcomes over sorafenib in advanced HCC, except for lenvatinib that shows a non-inferiority. Two other agents recently approved in patents: regorafenib and nibolumab showed better tumor response rate and durability as reported in one uncontrolled single-arm study.

1.8. Prognosis

Treatment outcomes in HCC are affected by multiple variables, including liver function, performance status of the patient and tumor stages. This relies on the importance of early diagnosis to receive curative therapies (surgery or ablation). Resection, generally has a satisfactory overall (OS) and disease-free survival rate (DFS) only in patients without liver cirrhosis, vascular invasion, normal liver function, good differentiation and early stages [111,112]. Indeed, the reported OS and 5 year DFS are 38.5-53.0% and 29.4-34.2%, respectively [111,113].

As the first-line therapy for unresectable BCLC B HCC, the overall median survival of patients receiving TACE remains dismal. It was reported that BCLC-B group of patients have 8 months OS rate and even lower to only 6 months in BCLC-C and Child-Phugh B patients [114]. The partial response was reported in 15-55% of patients, even though the reported five-year survival rates of TACE have only ranged from 1-8% [115,116]. Sorafenib SHARP trial conducted in patients with no prior systemic therapy showed a median OS of 10.7 months with sorafenib-treated patients in comparison to placebo. However, the tumor response rates were low, with no cresponse and partial response.

In conclusion, the HCC treatment solely relies on the early diagnosis, as the current treatment landscape, even improved by years, are still insufficient to provide a significant benefit towards patient in advance stages of the diseases. This issue puts further emphasis on the value of better diagnostic and predictive biomarker and alternative systemic therapies for a better patient's management.

2.1. MicroRNAs

In the recent past, non-coding RNAs (ncRNAs) have been identified as important regulators of many cellular pathways. In the last couple of decades, the existence of various types of functional ncRNAs were discovered, altogether with the technological advancement in the next generation sequencing tecniques and bioinformatics analysis that revealed their specific role in gene regulatory networks. Subtypes of non-coding RNAs are classified based on thelength of nucleotide chain, such as the long ncRNAs (>200 nucleotides), short ncRNAs (<200 nucleotides), microRNAs (miRNAs) (18-25 nucleotides). Some species are highly conserved ncRNAs such as microRNAs (miRNAs), and circular RNAs (circRNAs), while others, mostly long ncRNAs (lncRNAs) lack conservation across species. For the highly conserved ncRNAs, abundant studies have tried to reveal their holistic role in the complexity of regulatory networks in humans, especially in diseases.

Since its discovery in *C. elegans* in 1993, miRNAs became the most studied ncRNAs class in the fields of biomedical science [117]. miRNAs are a class of highly conserved short non-coding RNAs, 18 to 25 nucleotides long that regulate the expression of various genes, up to more than 60% of protein-coding genes [117,118]. They participate in the post-transcriptional gene silencing by targeting mRNAs, blocking the translation or even inducing their degradation. MiRNAs participate in the regulation through a synergic system as one particular miRNA can regulate more than one protein-coding gene, while one single mRNA can be targeted by multiple species of miRNA [119]. It is well eshtablished now that miRNAs have essential roles in various cellular processes, including the ones related to cancinogenesis, such as cell proliferation, differentiation, and apoptosis [120,121]. Currently, at least 2654 human mature miRNA sequences are registered in the official miRNA database miRBase release 22.1 [122].

It was only in 2002 that miRNAs made a debut in the medical research fields being associated to various pathological conditions, including cancer [123]. Abundant miRNA profiling studies described the significant difference in miRNA expression between tumor and healthy tissues. The relationship between decreased miRNA expression and the up-regulation of oncogenes in cancer cells indicates that miRNAs can function as tumor suppressor genes [124]. On the contrary, when the up-regulation of a specific miRNA is linked to the silenging of known oncosuppressor genes, the miRNA is considered as an oncomir [125,126].

2.2. Biogenesis of miRNA

The maturation of miRNAs undergoes several critical steps, including their transcription, processing by Drosha and Dicer, their loading onto Argonaute proteins and the final mRNA targets **(Fig. 8)** [127,128]. The coding genes of miRNA are interspersed in the genome, either within the introns or exons of protein-coding genes or in intergenic regions [127]. Most miRNA genes are transcribed by RNA polymerase II, originating from a primary transcript (pri-miRNA) that contains hundreds or thousands of nucleotides [129]. Pri-miRNAs are then processed to a miRNA precursor in the nucleus (pre-miRNA), 60-70 nucleotides in length with a stem-loop structure, by microprocessor complex that consists of the nuclease Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) [130]. Subsequently pre-miRNA are transferred to the cytoplasm with the help of the RAS-related nuclear protein with bound GTP (RAN-GTP)-dependent transporter exportin-5 (XPO5) [129]. In the cytoplasm, pre-miRNA is cleaved by Dicer, liberating a small 21-24 nucleotides length RNA duplex, consist of the so-called driver and passenger strand (miRNA/miRNA*) [131,132]. The guide strand of the RNA duplex, identified as the strand with the weakest base pairing at its 5' terminus, will be incorporated into Argonaute (Ago) protein, forming the RNA Induced Silencing Complex (RISC) [132]. This complex will mediate gene silencing either by translational repression or by promoting the degradation of target mRNAs in 3' untranslated region (3'UTR) [117,119]. Recently, it is discovered that miRNA can also be associated with any position of target mRNAs, or interfering with the expression of regulatory proteins or by altering DNA methylation and histone modification [129,133,134]

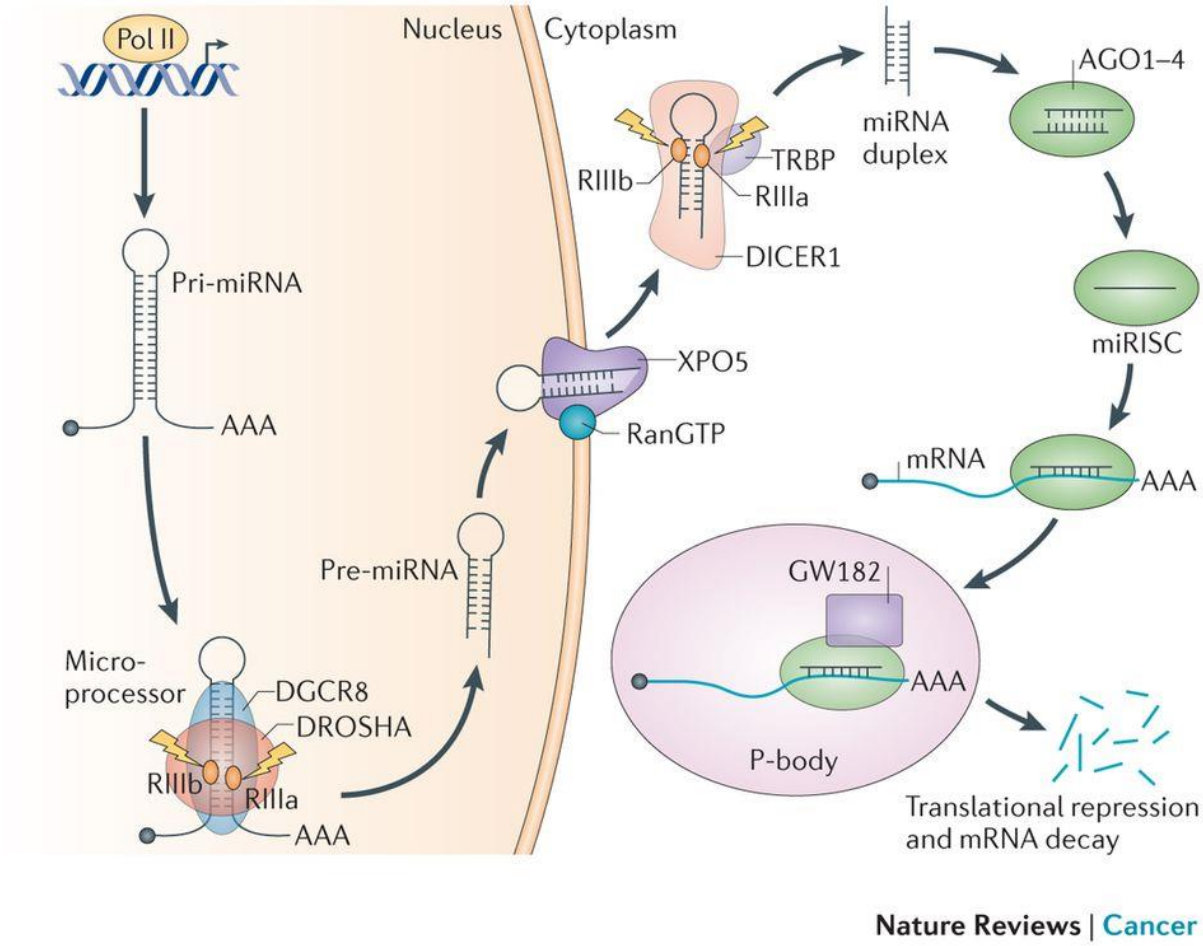


Figure 8. Biogenesis of miRNA. MiRNAs are transcribed by RNA polymerase II into a pri-miRNA precursor, which is subsequently processed by the enzymatic complex composed by DROSHA and DGCR8 into a pre-miRNA. The hairpin precursor is then translocated into the cytoplasm by Exportin 5 where is cut by Dicer complex and loaded to the Argonaute complex for the mRNA targeting. Taken from Lin and Gregory *et al.,* 2015 [128]

2.3. MiRNA in Human Diseases

Over the years, the research of miRNAs has thrived in the territories of diseases. The keyword of "miRNA" currently provide 89.000 results in PubMed, while the combination of "miRNA human disease" matches to 19.000 results in 2019, roughly showing the range of studied miRNA in the context of human diseases. The alteration of miRNA expression provided a premise of disease-specifics reflecting the pathological changes of vital biological processes. MiRNA-related studies mainly focused on high-priority human diseases such as cancer, viral infections, immune-related disorders and neurodegenerative diseas. From these observations, it seems ideal to use miRNAs as a biomarker or molecular therapeutic target,

or even merely to reveal the complete pathogenesis of specific key event in particular diseases.

2.4. MiRNA in Cancer

MiRNAs are aberrantly expressed in a variety of cancer, as the over- or under-expression of miRNAs will alter the expression of their target mRNAs on either down- or up-regulating the protein products. Due to this reason, a classification of miRNA in cancer might have a dual role either as an oncogene or a tumor-suppressor. Global miRNA profiling studies have revealed the plausible involvement of miRNA with several critical steps of cancer biology, ranging from cell cycle, apoptosis, invasion, angiogenesis, autophagy, metastasis, until drug-uptake pathway that leads to resistance to treatment. Moreover, it is well known that the alteration of miRNAs in cancer settings might involve multiple miRNAs instead of a single one, adding more complexity to the cellular network of cancer biology.

Despite the vast information regarding the biological role of miRNA on regulating gene expression, the understanding of how miRNAs are regulated remains incomplete. Several findings reveal that the most probable mechanisms of miRNA deregulation in cancers consist of chromosomal abnormalities, transcriptional control changes, epigenetic changes, and defects in miRNA biogenesis machinery [135].

2.5. Chromosomal Abnormalities

A substantial amount of evidence supports that more than half miRNA genes are located in cancer-associated genomic regions (CAGRs) in the chromosomal fragile sites (FRA) that are predisposed to DNA instability in cancer cells, as it was a preferential site of chromatid exchange, translocation, deletion, or any integration of plasmid DNA and tumor-associated viruses [136]. Abnormal miRNA expression in malignant cells is often attributed to the alteration of genomic miRNA copy numbers in these locations (amplification, deletion or translocation) [135].

2.6. Transcriptional Control

Abnormal expression of miRNA in cancer could derive from the dysregulation of several key transcription factors that acts as a “tuning fork” for post-transcriptional regulation of miRNAs, thus leading to tumorigenesis. As an example, c-Myc, a proto-oncogene

transcription factor known for regulating 10-15% human genes, is well known to be deregulated in many cancers [132,137]. O'Donnell *et al*. (2015) reported the simultaneous alteration of miR-17-5p-miR-20a cluster and their target genes by c-Myc, resulting in a cell cycle progression [138]. *In vivo* analysis in lymphoma showed the direct activation of miR-17-92 by Myc through the direct binding of c-Myc to promoter region [139]. Besides c-Myc, p53, a potent tumor suppressor in cancer also modulates the levels of miR-34 by binding to a specific binding site within the gene, contributing to the increase of apoptosis [140].

2.7. Epigenetic Change

Epigenetic is defined as heritable changes in gene expression without a change of DNA sequence, consist of DNA methylation and histone modifications [141]. It is believed that miRNAs are also susceptible to epigenetic modulation [135]. Discovery by *Saito et al.* (2019) showed that DNA methylation and histone modifications could affect the expression of miR-127 in cancer cell lines after the exposure to 5-Aza-CdR, a potent DNA methylation inhibitor, and 4-phenylbutyric acid (PBA), a histone deacetylase (HDAC) inhibitor [142]. *Wang et al.* (2019) also observed that the decrease of methylation in the promoter region of miR-130b was associated with the elevation of miR-130b expression in lung cancer patients [143]. The above evidence highlighted the role of epigenetic regulation of miRNAs during tumorigenesis, even though its complete mechanism has yet to be understood.

2.8. Defects of miRNA Biogenesis Machinery

MiRNA biogenesis is controlled by several enzymes and regulatory proteins ranging from Drosha, Dicer, DGCR8, Argonaute proteins, and exportin-5. Therefore, mutation and abberant expression of one component could lead to the dysregulation of miRNA. A recurrent mutation affecting metal-binding residue of the RNAse IIIb domain (EE147K) of DROSHA, as well as non-recurrent mutations in other machinery, leads to the predominant downregulation of a subset of miRNAs that play key events in embryonal tumorigenesis [144]. The depletion of Dicer1 and Drosha by p53 tumor suppressor gene was also reported to enhance tumorigenesis *in vivo* by affecting global regulation of miRNA biogenesis in cancer [145].

2.9. MiRNAs in HCC

Since a decade ago, numerous miRNAs are identified as deregulated in HCC, broadly affecting almost all aspects of cancer biology from proliferation, apoptosis, invasion, metastasis, epithelial-mesenchymal transition (EMT), angiogenesis, autophagy, to the resistance towards HCC therapy. The dysregulation of miR-122, one of the most abundantly expressed miRNA in liver, has been reported in various studies to be associated with proliferation, apoptosis, and EMT in HCC [146–148]. Recently, miR-122 was also associated with resistance to doxorubicin and sorafenib [149,150].

In line with the fact that several miRNAs might regulate one single biological pathway, different miRNAs can regulate one main cancer pathway in HCC. Different miRNAs have been reported to target several key driver genes in HCC. It was described that HCV core protein enhance TERT protein expression through downregulating miR-138, which in turn inhibits HCC cells replicative senescence [151]. *TP53*, a pivotal tumor suppressor genes had been reported to strongly interact with miRNA. *P53* regulates the expression of several downstream miRNAs involved in key cellular processes such as proliferation and apoptosis while in the other end, miRNAs can modulate *p53* expression and activity by targeting its 3'UTR mRNA or indirectly inhibiting p53-modulator proteins [152]. Yang *et al.* (2016) performed a profiling analysis and identified a panel of 33 miRNAs, whose expression changed in *TP53* wild type HepG2 cells following doxorubicin treatment that induces p53 activation [153]. MiR-34 family has been reported to be deregulated in human HCCs, as it was reported that HCC patients and low miR-34a levels are associated with tumor metastasis and invasion [154]. However, there is an open question whether miR-34a is truly an oncomiR or tumor-supressor miRNA. In one hand, miR-34 is reported to inversely regulating *c-MET*, a strong proto-oncogenes involved in cell migration and metastasis. Gougelet et al. (2016) revealed that miR-34a might acts as an oncogene in HCCs carrying mutation in the β-catenin pathway. These finding calls further discoveries to validate the role of this miR in p53 pathways [155]. Several miRNA had been reported to directly or indirectly target *CTNNB1*, participating in β-catenin pathway in HCC. MiR-206, miR-885-5p and miR-214 were all found to be down-regulated in HCC tumor tissues [156–158]. Mir-214 indirectly targets *CTNNB1* via surpressing the enhancer of zeste homologue 2 (EZH2) and is associated with the invasion of HCC cells [158]. Thus, it is clear that miRNAs play a significant role on directly or indirectly regulating

driver mutation genes in HCC progression, underlying an importance of further profiling or validation studies to reveal their role.

When it comes to specific pathways, Vasuri *et al.* (2018) recently identified mTOR pathway as the most represented miRNA-regulated pathway in HCC. This observation arises from all the recent studies reporting the role of miRNAs in HCC tumorigenesis in cellular models, with most of the miRNAs acting as tumor suppressors and affecting the cell growth, survival, and metabolism of cancer cells [159]. Moreover, dysregulation of miRNAs also had been extensively studied in the relation to HCC metastasis. Great deals of miRNAs were reported to regulate angiogenesis in HCC by *VEGF* signaling pathway, one of the main mestastatic-related genes in HCC. Overexpression of miR-146a and miR-638 was showed to repress HCC angiogenesis by directly decreasing *VEGF* secretion. On the contrary, some miRNAs could enhance angiogenesis by *VEGF* [160,161]. Supression of miR-338-3p had been related to promote angiogenesis [162]. Up till now, numerous new studies had reported an extensive amount of miRNAs in relation to HCC pathways. The abberant expression of miRNAs in HCC was summarized in **figure 9**, indicating the prospect of miRNAs as a potential biomarker and potential therapy in HCC [163].

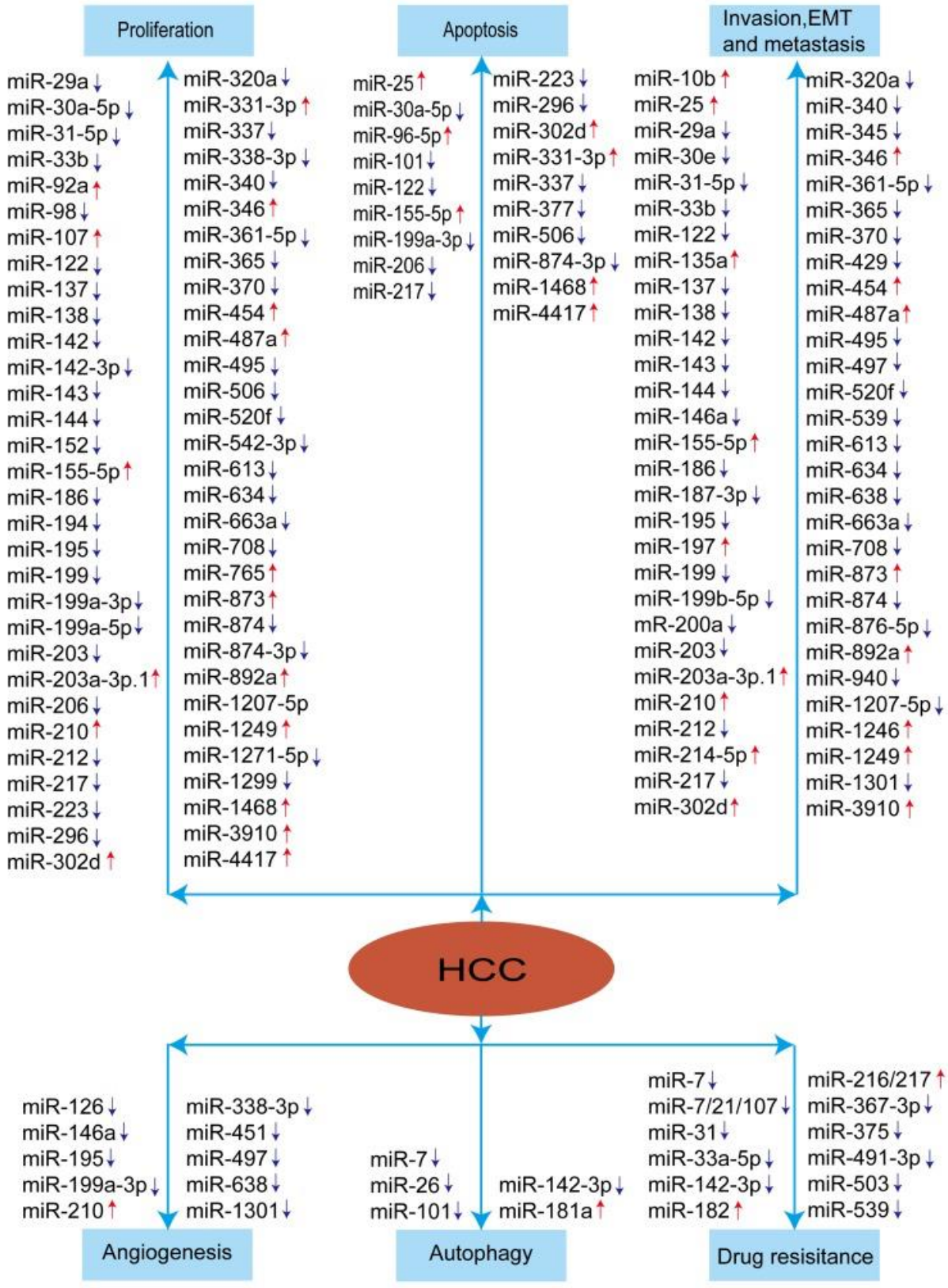


Figure 9. Summary of miRNAs in the development and progression of HCC. Red arrow means: increased expression of miRNA, blue arrow means: decreased expression of miRNA. Taken from Xu *et al.*, 2018 [163]

2.10. MiRNAs in Drug Resistance to HCC Therapy

In tumor resistant cells, several mechanisms participate in the development of drug resistance in HCC. Recently, it was shown that altered expression of subsets of miRNAs plays an essential role in regulating genes involved in cell apoptosis, cell proliferation, autophagy, epithelial-mesenchymal transition (EMT), and regulation of drug efflux. Although these mechanisms have been previously investigated, the role of miRNAs remains ambiguous and unclear. However, there were several *in vitro* studies reporting the involvement of specific miRNAs in each different pathway related to drug resistance in different HCC cell lines, suggesting their essential role as a critical player in both anthracyclines and sorafenib resistance **(Fig. 10 a and b).**

One of the most critical mechanisms of dox resistance consists of drug elimination by the transporter family known as the ATP-Binding Cassette (ABC) transporters, known to be highly abundant in hepatocytes [164]. *ABCB1*, also known as Multidrug Resistance 1 (MDR1) or P-glycoprotein (P-gyp), is expressed in 80-90% of HCC cases [165–167] where it lowers the intracellular drug accumulation of working as a pump actively extruding exogenous compounds out of cells [165,166,168]. The overexpression of miR-122 induces cell cycle arrest that leads to the down-regulation of multidrug-resistant (MDR) genes, *ABCB1*, and *ABCF2* [165,169]. *ABCB1* gene is also directly targeted by miR-223 in both mRNA and protein levels, and the overexpression of miR-223 can sensitize HCC cells to dox [170]. Moreover, miR-375 is also able to decrease the expression of *ABCB1* by targeting Astrocyte Elevated Gene-1 (AEG-1) and thus participating in dox resistance [171–173].

Autophagy is an adaptive mechanism promoting cell homeostasis by the degradation of useless and damaged proteins or other cytoplasmic components in the lysosomal system [174,175]. However, in tumor cells, autophagy will also maintain metabolic homeostasis when cancer cells are subjected to stressful environments such as nutrient deprivation, hypoxia, or drug-induced damages during chemotherapy or targeted therapy [176]. One of the systems involved Autophagy-related Gene 7 (ATG7) that is targeted by miR-375, preventing the maturation of authophagosome thus inhibiting autophagy [177]. The role of autophagy during treatment with sorafenib is paradoxical. Some studies have established that sorafenib–induced autophagy is a cellular adaptive mechanism that promotes the survival of cancer cells [178]. Thus inhibition of autophagy will enhance the effect of the drug [179].

On the contrary, sorafenib was reported to inhibit the pro-death role of dox-induced autophagy in Hep3B cells [180–182]. In sorafenib-resistant HCC cells, miR-142-3p was able to sensitize cells to the drug by inhibiting the drug-induced autophagy [183]. Additional studies described the correlation between miRNA and autophagy-related sorafenib resistance; miRNAs such as miR-153, miR-216a, miR-217, miR-10a-5p, miR-222, negatively regulate *PTEN*, leading to the overexpression of *AKT* and *mTOR*, regulating autophagy, proliferation, and apoptosis [184–186].

Apoptosis is one of the main pathways involved in the MDR. In this pathway, one of the key regulators is the tumor suppressor p53 transcription factor that is activated in response to various cell stress and behavior, including DNA damage. The induction of DNA damage from chemotherapeutic agents may lead to cell cycle arrest, DNA repair, or apoptosis through the p53 pathway [187]. Jun *et al*. (2014) reported that the up-regulation of miR-182 increased resistance to cisplatin, by inhibiting the expression Tumor Protein P53 Inducible Nuclear Protein 1 (TP53INP1), a pro-apoptotic gene of the p53 pathway, leading to the increase of cell viability during treatment with cisplatin [188]. Another miRNA involved in the anti-apoptotic mechanism is miR-101 that has a tumor suppressor role in HCC cell lines. MiR-101 acts as a negative regulator of Mcl-1, a critical anti-apoptotic protein in cancer cells that maintains crucial elements in tumor environments such as growth factor and cellular stress [189]. Mcl-1 has been associated with several drug-resistance phenomena in various cancers [190], and its downregulation by miR-101 suggests potential application for this miRNA in a dox resistance setting [189]. During sorafenib treatment several miRNAs were described to dysregulate the apoptotic pathway contributing to the resistance. MiR-122 for example, was also significantly reduced in sorafenib-resistant cell lines [191]. The mechanism by which this liver-specific miRNA reverses drug resistance involves the downregulation of insulin-like growth factor 1 receptor (IGF-1R) that was proven to inhibits apoptosis and disrupt tolerance to sorafenib *in vitro* [191].

Several miRNAs play an essential role in the mechanisms of tumorigenesis like the invasion and proliferation of drug-resistant cells. Up-regulation of miR-181b in dox-resistant HCC cell lines leads to the inhibition of TIMP Metallopeptidase Inhibitor 3 (TIMP3), an inhibitor of cell migration, invasion, and angiogenesis in cancer cells [192]. In addition, miR-199-3p represses the translation of mTOR and c-Met, suggested as a major activator of MMP, resulting in a reduced cell invasion and metastasis of dox-resistant cells[193].

Several microRNAs are also regulating the P13K/AKT pathway that is involved in various regulatory mechanisms in HCC, such as cell proliferation, invasion, apoptosis, metastasis, and autophagy during cancer progression. Kabir *et al*. (2017) identified miR-7 as a potent tumor suppressor targeting Tyrosine-Protein Kinase 3 (TYRO3) that regulates proliferation, migration, and invasion through the P13/AKT pathway in sorafenib-resistant Huh-7 cells model [194]. Another study showed that the overexpression of miR-494 in HCC leads to the decrease of *PTEN*, a multifunctional tumor suppressor that will activate P13/AKT signaling pathway and promoting anti-apoptosis and anti-proliferation in HCC [195,196].

Hypoxia plays a significant role in enhancing the resistance of cancer cells to cytotoxic drugs [197], particularly relevant in the setting of concurrent induction of acute hypoxia during TACE [168]. The hypoxic environment, to which surviving cells are exposed during and soon after chemoembolization, lead to the adaptive mechanisms in tumor proliferation, metabolism, and angiogenesis, that involve HIF-1 [198]. miR-338-3p was observed to be down-regulated in HCC patients and strongly associated with resistance to sorafenib. The re-exposure of miR-338-3p to resistant-cell models re-sensitizes cells by targeting *HIF-1α*, known to elicit the hypoxic environment in resistant tissue, by enhancing angiogenesis, metabolism, and resistance to apoptosis [199].

The involvement of microRNAs on numerous regulatory networks and mechanisms of drug resistance and the remarkable specificity of the circulatory expression patterns in cancer settings has raised provocative questions regarding their potential as markers to predict resistance in HCC.

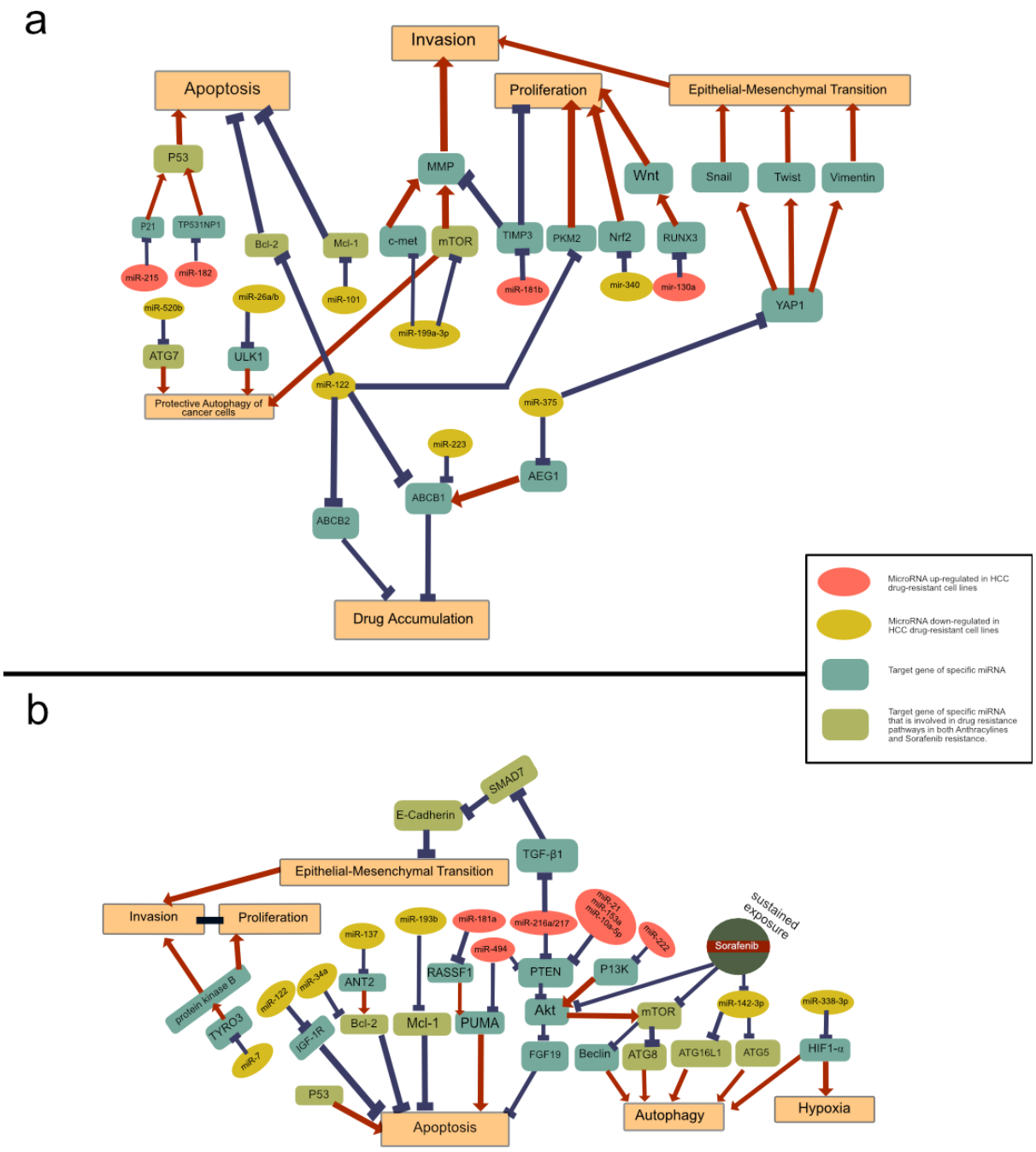


Figure 10. Molecular Pathways of miRNAs involved in a) doxorubicin resistance; b) sorafenib resistance in HCC. (Pratama *et al.,* 2019) [200]

2.11. Circulating MiRNAs

In 2008, Mitchell *et al.* (2018) observed a panel of specific miRNAs in human plasma that is remarkably stable from endogenous RNAse activity and originating from human prostate cancer xenografts [201]. This discovery has extended the possibility of analyzing

circulating miRNAs as indicators of the current state of a disease. Regarding cancers, numerous studies have demonstrated that miRNAs are released into the bloodstream, through cell lysis such as tumor apoptosis and necrosis, and also through active secretion [202]. The stability of circulating miRNAs is influenced by the presence of agonaute-2 (Ago-2), a key effector protein, as a carrier in peripheral blood. High-density lipoproteins (HDL) also shown to participate in the transport and delivery mechanism of miRNAs in human plasma [202,203]. Interestingly in blood and serum, circulating miRNAs are not digested by RNAse, resist from degradation in different pH ranges, nor affected by changes of temperature [202,203]. Indeed, the presence of highly conserved miRNAs can also be found in other forms of body fluids such as saliva, cerebrospinal fluid, ascites, urine and semen [202,203]. From this knowledge, it is assumed that miRNA released by their producing cells will remain stable for a long period in biological fluids on such protected form, making them suitable as a biomarker of diseases [204].

2.12. Circulating MiRNAs as diagnostic and prognostic biomarkers in HCC

A clinical biomarker has been defined as "an objective and quantifiable characteristic of biological processes in human that might consist of any cellular, biochemical, molecular, or genetic alteration, indicating a broad range of physiological state, pathogenic processes, pharmacologic responses, or therapeutic intervention" [203–205]. The implication of biomarker discovery has been employed from basic to clinical research to diagnose and predict outcomes of diseases as well as guides of treatment. Studies of biomarker itself have been encouraged, as they are considered as less invasive methods for the development of precision medicine [205]. Indeed, the prospect of discovering a reliable biomarker, especially in cancer studies where non-invasiveness and accurateness are looked at as significant pitfalls, has gained a great attention [203]. Thus, the characteristic of circulating miRNAs holds a potential value as clinical biomarkers, due to both their highly conserved nature and stability in circulation.

Abroad range of studies conducted in different types of cancer reported circulating miRNAs to be dysregulated at early stage of cancer or even before the confirmation from clinical symptoms, imaging examinations or biopsy [203,205]. As an example, blood-based miR-126-5p was able to diagnose the aggressive subtype of triple-negative-breast cancer (TNBC) in the early stages of disease [206]. Six miRNAs (let-7b-5p, miR-192-5P, miR-19a-3p,

miR-19b-3p, miR-223-3p, and miR-25-3p) from serum are able to diagnose pancreatic cancer, one of the deadliest type of cancers in the early stage [207].

In addition, Souza *et al.* (2019) reported a panel of serum miRNAs differently expressed in each of molecular subtypes of breast cancer with subtle sensitivity and specificity value, potentially to be used in the future as an early detection biomarker of breast cancer [208]. In consideration of these examples and the many other eports present in literature, circulating miRNAs have a high potential as an alternative biomarker to diagnose, discriminate clinical stages in cancer, preventing unnecessary invasive technique such as biopsy on suspected patients

In HCC, several studies over the years have reported the potential of circulating miRNAs as a diagnostic and predictive biomarker, considering the insufficient sensitivity or specificity of current diagnostic techniques and the failure of available therapies in the clinical setting. Different profiling studies from various parts of the world were able to discover a panel of miRNAs distinguishing HCC based on the most common risk factors within the study population. In East Asia, where chronic hepatitis B is the most common risk factors, circulating miR-126, miR-224, miR-125b, miR-21 are reported to be able to distinguish HCC from chronic hepatitis B infection, thus suggesting their potential as an early diagnostic biomarker for this setting [136, 138]. On the other hand, miR-21, miR-199a, miR-203, miR-122, and miR-301 represent an example of differently expressed miRNAs between the group of HCC patients and chronic hepatitis C, in high endemic population like Egypt [132, 143, 147]. Circulating miRNAs are also reported to be differently expressed between cirrhotic and HCC patients regardless the etiologies; putting on mark that early prediction and diagnosis are important milestones to achieve a successful treatment in HCC, these reports might serve as a significant potential to address this issue [144, 148].

The potential of circulating miRNAs as a predictive and prognostic biomarker after therapy have also been reported in different treatment setting. For curative treatments, two separate studies reported the potential of circulating miR-1246 as a predictor of tumor recurrence in both post-resection and post-transplantation patients [135, 145]. Moreover, other studies have also reported the potential of circulating miRNAs in non-curative therapies. Two Korean cohorts have reported the potential of plasma miR-122, miR-21, miR-26a, and miR-29a-3p predict early refractoriness in HCC patients after TACE [140, 146]. In the setting of systemic therapy for advance HCC, Teufel et al. (2015) has recently reported the

association of a panel of plasma miRNAs with overall survival after regorafenib, serving a potential of these panels to identify HCC patients most likely to respond to regorafenib [214]. Two different cohorts from Japan and Italy have also reported the potential of circulating miR-181a-5p and miR-425-3p to predict therapy response after sorafenib. However, these reports need more validation in larger sample size [215,216]. The summary of every profiling studies of circulating miRNAs in HCC over the past three years (2017-2019) is summarized in **table 2.**

Studies	miRNAs	Up/down-regulation	Samples	Subjects
Amr *et al.*, 2016.[217]	Mir-21	Up	Serum	HCC *vs* Chronic Hepatitis C
	MiR-199-a	Down		
Khairy *et al.*, 2016.[210]	MiR-203	Down	Serum	HCC *vs* Non-HCC (Treatment-naïve chronic HCV and HCV with cirrhosis)
Hung *et al.*, 2016. [218]	MiR-122 Let-7b	Up	Serum	HCC vs Dysplastic Nodule
Wang *et al.,* 2016[219]	MiR-148a MiR-148b Mir-152	Down	Serum	HCC *vs* Benign Liver Disease *vs* Healthy Controls
Ng *et al.,* 2016.[212]	MiR-148a MiR-1246	Up	Serum	Post-transplantation HCC recurrence *vs* no-recurrence patients
Ghosh *et al.*, 2016 [209]	MiR-126	Up	Plasma	HBV-HCC *vs* HBV-non-HCC
Okajima *et al.*, 2016 [220]	MiR-224	Up	Plasma	HCC *vs* Healthy Control

Lin *et al*., 2016 [221]	Mir-224	Up	Serum	HCC *vs* Chronic HBV vs Healthy Control
Zhang *et al*., 2017 [222]	MiR-92a-3p Mir-107 Mir-3126-5p	Up	Serum	HCC *vs* healthy control
Kim *et al*., 2017.[213]	MiR-122	Up	Plasma	TACE-refractory HCC patients
Nishida *et al*., 2017.[215]	Mir-181-5p	Up	Serum	Partial Responder *vs* Stable *vs* Progressive Disease post Sorafenib
Chen *et al*., 2017.[223]	Mir-125b	Down	Plasma	HBV-HCC *vs* Chronic HBV
Guo *et a*l., 2017. [224]	MiR-21	Up	Serum	HCC *vs* Chronic HBV
Ali *et a*l. 2017.[225]	MiR-122	Up	Serum	HCC *vs* Chronic HCV
Shehata *et al.,* 2017. [226]	MiR-34a	Up	Serum	HCC *vs* Liver Cirrhosis vs Healthy control
Moshiri *et al*., 2018.[227]	MiR-101-3p MiR-106b-3p MiR-1246	Up	Plasma	HCC *vs* Liver Cirrhosis vs Healthy Control
Kim *et al*., 2018. [228]	MiR-21 MiR-26a MiR-29a-3p	Up	Plasma	TACE-reftactory HCC patients
Mourad *et al.,* 2018 [229]	MiR-16 MiR-34a	Up	Serum	HCC *vs* Chronic HCV *vs* Liver Cirrhosis

	MiR-221			
	MiR-125a MiR-139 MiR-145 MiR-199a	Down		
Weis *et a*l., 2019. [211]	MiR-486-5p	Up	Serum	HCC *vs* Liver Cirrhosis
	MiR-122 MiR-142-3p	Down		
Chuma *et al*., 2019. [230]	MiR-1246	Up	Serum	Early tumor recurrence (ETR) HCC *vs* without ETR
El-Hamouly *et al*. 2019.[147]	Mir-301	Up	Plasma	HCC *vs* Chronic HCV
Ning *et al*., 2019 [231]	MiR-155 MiR-96	Up	Serum	HCC *vs* healthy control

Table 2. Current profiling studies of miRNAs in HCC from 2017 to 2019

However, there are a significant amount of disadvantages and limitation that needs to be considered to utilize miRNAs as significant biomarker in clinical setting. One of the major limiting factors is associated with the fact that one particular miRNA may target multiple different mRNAs, consequently may play different, opposite, and have significant alteration in more than one type of cancer. For example, the up-regulation of circulating miR-21 has been found in patients with colorectal, lung, breast, prostate, liver, esophageal and endometrial cancers [232], in such way might be a significant drawback on utilizing miR-21 as an early diagnostic or surveillance marker of specific cancer in patients with unspecific symptoms. Moreover, inconsistencies may also vary among the very similar studies in same

diseases. Mir-148a for example, has been reported both to be upregulated and downregulated in two studies of HCC setting from two different population [167,219]. Those inconsisten observations could derive from the heterogeneity in the miRNA expression patterns and from the different molecular subtypes of cancer[33]. Moreover, recent evidences suggest that individual variability, such as race and gender, as well as external factors and life-styles, drug consumption, smoking habits, diets, and most importantly, different etiology and risk factor of cancers, could contribute to affect miRNA level in circulations [211,218,233,234]. Inter-individual variables, such as race, gender and age, are also suggested to influence the consistensies during the profiling studies of circulating miRNA. Thus, it is essential to consider the report of significant miRNAs only between studies with minimum interindividual or individual variability, probably by stratifying the result based on the shared etiological risk factor, ethnicities, and stages of disease. Considering these issues, profiling and validation studies for circulating miRNAs till need to be refined by studying well characterized and large cohorts of patients.

As a circulating biomarker to predict response after therapy, one miRNA candidate need to be validated whether they might be just results or by-products of diseases or indeed participated in the occurrence and development of tumors both directly or indirectly. Indeed, the miRNA candidate needs to be further elucidated whether it is participating in the resistance towards one particular treatment. Several studies have reported fluctuations in circulating miRNA levels in response to chemotherapy, thus marking the potential of specific alteration patterns of miRNA before and after therapy. However, there are no available studies in HCC reporting the differential of expression of circulating miRNA following a large cohort of patients on more than one time point across the treatment regimen, more importantly, before, shortly, and long-term after treatments to evaluate the pattern of circulating miRNA.

When it comes to technical and methodological drawbacks, it is vital to use the same type of material (for patients and control), as circulating miRNA levels were reported to be higher in serum than in plasma, implying a potential interference by platelet and white blood cell during sample presentation. It is also essential to use a standardized protocol of sample collection and processing, something that is still not yet validated in miRNA studies. A general rule to use strictly controlled procedure in the way samples are collected and prepared (blood withdrawal and serum preparation) and processing (RNA extraction and analysis) might be

the best way to prevent the technical issue. Measurement of hemolysisi, internal controls (positive and negative) to monitor technical/biological issues is also essential. Post-analysis, miRNA expression needs to be normalized by a reference gene for miRNA research to correct a systematic bias, which unfortunately has not reach a universal consensus up to today. Nevertheless, this highlights the importance of standardizing every aspect of circulating miRNA analysis to avoid unspecific variations that could hamper the clinical application of even the most promising miRNA biomarker.

Chapter 2

Aims of the Study

Hepatocellular carcinoma (HCC) represents the fourth leading cause of cancer-related death worldwide due to the late diagnosis and its poor prognosis rate. Non-invasive diagnostic and predictive biomarker might be a crucial strategy to support the idea of individualized treatment in order to improve the prognosis of HCC patients. In the present study, we conducted a longitudinal study from multiple cohort of patients to address the potential of circulating miRNA as a non-invasive biomarker for HCC, as well to assess its molecular role in HCC, with several main objectives consist of:

- Identify circulating miRNAs as predictor of early HCC occurrence in high-risk population setting, specific in DAA treated chronic HCV patients.
- Identify circulating miRNAs as a prognostic non-invasive biomarker after HCC treatments.
- Identify putative targets of each potential miRNAs and their cellular involvement in HCC pathway by *in silico* target prediction methods and *in vitro* approaches using miRNA transfection methods in HCC cell model.

Chapter 3

Materials and Methods

3.1 General Procedures

3.1.1. Serum Collection

Serum samples were obtained from 10mL of whole blood collected in sterilized and centrifuged at 3000 rpm for 10 minutes in an Allegra 25R^{TM} (Beckman Coulter, CA, USA) centrifuge. Supernatants were transferred in 1mL eppendorf tubes and subsequently frozen at -80°C for long-term storage.

3.1.2. Circulating miRNA Isolation and Quantification

Small RNAs were isolated from 300uL of serum using the NucleoSpin™ miRNA Plasma Kit (Macherey-Nagel, Germany). MicroRNAs were quantified in a Qubit® 2.0 Fluorometer (Thermo Fischer Scientific, Waltham, MA USA) by using the Qubit microRNA Assay Kit (Thermo Fischer Scientific, Waltham, MA USA) following the manufacturer instructions.

3.1.3. Microarray Profiling

Small RNAs were labelled with the FlashTag™ Biotin HSR RNA Labeling Kit (Affymetrix®, Thermo Fischer Scientific, Waltham, MA USA) and hybridized on Genechip miRNA 3.0 (Thermo Fischer Scientific, Waltham, MA USA) containing 1,734 human mature miRNAs. The array cartriges were processed on an Affymetrix Fluidic Station 450 and scanned on an Affymetrix GeneChip 3000 7G. The robust Multichip Analysis (RMA) algorithm was used to derive CEL file probe-level hybridization intensities at the gene expression levels.

3.1.4. Reverse Transcription for miRNA

Thirty nanograms of small RNAs were reverse transcribed by using the qScript microRNA cDNA Synthesis Kit (Quantbio, Beverly, MA USA). Two-steps methods were used for reverse transcriptase of small RNAs:

1. Poly(A) tailing

Small RNAs samples were mized with 2ul of 5x reaction buffer, and 1 ul of Poly(A) polymerase enzyme. Using T100™ thermal cycler (Biorad, CA USA), samples were incubated for 60 minutes in 37°C, and 5 minutes in 70°C.

2. cDNA synthesis

Poly(A) reaction were then mixed with 9 ul of qScript cDNA reaction mix, and 1ul of ReadyScript reverse transcriptase enzyme. Using T100™ thermal cycler (Biorad, CA USA), samples were incubated for 20 minutes in 45°C, and 5 minutes in 85°C.

3.1.5. Quantitative Real Time PCR (qPCR) for miRNAs

Per PCR reaction, 0.15 ng of cDNA samples were mixed with 1X PerfeCTa SYBR® Green SuperMix (Quantabio, Beverly, MA), universal primer (200nM) (Quantbio, Beverly, MA), miRNA PCR Primer (200nM) (Metabion, Germany), and nuclease free water to reach the final volume of 25µL. Samples were run in duplicate. PCR reactions were run in a CFX-96 thermal cycler (Bio-Rad Laboratories, Hercules CA) in a two steps cyclin protocol consisting of activation (2 minutes, 95°C), and 45 cycles of denaturation (5 seconds, 95°C) and annealing (30 seconds, 60°C followed by the melting curve analysis. The Cq for each miRNA was obtained by calculating the arithmetic mean average of duplicates in a 25µL reaction. Cq values >40 were considered as negative, and the melting point curves were observed for all assays to verify primer specificity. The relative quantification was obtained using the Pfaffl modification of the ΔΔCq equation, taking into account the efficiencies of individual genes and results were normalized to miR-1275 and miR-1280. All primes were purchased from Sigma Aldrich (St. Louis, MS, USA).

3.1.6. RNA Extraction, quantification and quality assessment

RNA from tissues or cells were extracted using TRI-Reagent Kit according to manufacturer's instructions. Samples were lysed with the reagent, chloroform was added and cellular RNA was precipitated by isoprophyl alcohol. After washing with 75% ethanol, the RNA pellet was dissolved in nuclease-free water and stored at -80 C until further analysis. The total RNA concentration and the purity were assessed by spectrophotometric analysis in a Beckman DU730_spectrophotometer (Beckman Coulter, CA, USA). The integrity of RNA was assessed on standard 1% agarose / formaldehyde gel.

3.1.7. Reverse Transcription-qPCR for Gene Analysis

Total RNA (1ug) was reverse-transcribed using the i-Script™ cDNA Synthesis Kit (Biorad, CA USA) in a T100™ thermal cycler (Biorad, CA USA) in agreement with the reaction protocol proposed by the manufacturer: 5 min at 25°C (annealing), 45 min at 42°C (cDNA synthesis) and 5 min at 85°C (enzyme denaturation). Real Time quantitative PCR was performed using the CFX-96 thermal cycler (Bio-Rad Laboratories, Hercules CA, USA). All primers pairs were synthesized by Metabion (Metabion, Germany) and were designed using the software Beacon Designer 7.91 (PREMIER Biosoft International, Palo alto, CA USA), β-actin and 18s were used as reference genes for gene analysis.

PCR amplification was carried out in 25µL reaction volume containing 25ng of cDNA, 1x iQ SYBR Green Supermix [100 mM KCl; 40mM Tris–HCl, pH 8.4; 0.4mM each dNTP; 50U/mL iTaq DNA polymerase; 6mM MgCl2; SYBR Green I; 20nM fluorescein; and stabilizers] and 250nM gene specific sense and anti-sense primers and 100nM primers for 18S. Standard curves using a "calibrator" cDNA (chosen among the cDNA samples) were prepared for each target and reference gene. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Non-specific products of PCR were not found in any case. The relative quantification was made using the Pfaffl modification of the ΔΔCt equation, taking into account the efficiencies of individual genes. The results were normalized to 18S and beta-actin.

3.1.8. PCR Primer Designs for Gene Analysis

All primers pairs were synthesized by Metabion (Metabion, Germany) and were designed using the software Beacon Designer 7.91 (PREMIER Biosoft International, Palo alto, CA USA), β-actin and 18s were used as reference genes for gene analysis. All the sequences of primers are listed in **Table 3.**

Gene	Forward primer	Reverse primer
NLRP3	CAGGAAGATGATGTTGGA	CCGACAGTGGATATAGA
NDEL1	AAGACAACAGGAAGTAACTA	AAGAAAGTGATGCTTGGA
JMJD8	CCTTCCTCTTCTCATCCA	CAGCACTCAACTCTTCAC
NCOA1	GGATTAGATGTATTATCAGAGA	AGAAGGAGAAGAGTAAGG

TIMP2	AAGGAAGTGGACTCTGGAA	CTTTGAACATCTTTATCTGCTTGA
HIF3A	GAGTATCGTCTGTGTCCATT	AGAGTGTTGCTCCGTTTG
CD274	CGACTACAAGCGAATTAC	TGTCAGTTCATGTTCAGA
NUPR1	ACTTATTCCCGCTGACTG	CTCTCACTCCCCATCTTG
TRAF4	GTAATAAGCACCGACACT	GCCTCATTAACTCTTCTCTA
IGFBP5	AATTGTGACCGCAAAGGATT	TACTTGTCCACGCACCAG
CDKN2D	GGGTTATGTATCAGAAGAGA	AACACCTATAAGCCACAA
FOXP2	GAGATTCAGCAGTTATGG	ATTGTTAGTAGTGAGGTCTA
MXD1	GATGAACATCCAGATGCT	TGTCCTTGTTATTGTATGGT
HOXC6	AGAATGTCGTGTTCAGTT	TGTTATGTCCTAAGGTGTT
ERCC1	AGGAAGAAATTTGTGATAC	TGTGTAGATCGGAATAAG
CREBL2	GAACTCAAGCAGGCATAC	CTTCAATCACTGACTCATCTT
DUSP9	AGAAGAATGGTGACTTTC	CATCAATGAACTCAATGG
SKIL	TTCCACCAGTTCTCTTCT	ACATAACAACACATTCTTCTTC
MSR1	AGAAGAGAATCCAGCATA	AAGCAATGTGGTATTCAA
KLK4	ACTGAGTGGATAGAGAAA	GAACAGATATTCCTGAATT
CTNNBIP1	TGACCAACAGAAACCTTT	AATCAGACCTCTTCACATT
BAG5	CTTCTATTAGTAGGCTTCAG	TCTTGTAATTCTTGTCATCT
DLG5	TACAGCAGGTACTTCACA	TTGTTCTTGATTGACCATTG
EIF4A2	GCACCACATATTGTTGTT	TTCATCTGCTTCATCCAA
RFLP3	GAAGATTCTACAGATGAACC	AATGAGGAGGAAGTTGTT

Table 3. Primer sequences of genes used in this study.

3.1.9. General Statistical Analysis

Statistical analysis were performed using NCSS 11 software (2016). (NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss). All Data were normalized and outliers are removed for further analysis. Pairwise comparison between groups were analyzed using non-parametric Mann-Whitney U test. For multiple comparison of variables, the Kruskal-Wallis one way ANOVA test were used. The friedman test were used to analyze the expression of miRNA between each time points. The receiver operating characteristic (ROC) curves were

used to estimate the discriminatory potential of the miRNAs. Kaplan-Meier survival analysis was used to estimate the potential of significant miRNAs to predict overall survival after treatment.

3.2. TASK 1 - Serum miRNA biomarkers for early hepatocellular carcinoma occurrence following direct-acting antivirals treatment

3.2.1. Patient Characteristics

We conducted a real-life-practice observational study on prospective out-patients treated for chronic HCV infection with DAAs from January 2015 to December 2016. Recruitment of patients is carried out in Padua University-Hospital liver centers and the major objectives of the study were: a) to investigate the development of *de novo* liver cancer after DAAs therapies and b) to identify miRNA profiles that predicts early emergence of HCC after DAAs. Thus, our study has been focused on comparison of 40 matched cirrhotic subjects with and without development of HCC after DAAs therapy. A total of 206 cases were treated with DAAs and according to the exclusion criteria, 36 cases with F3 fibrosis staging (no cirrhosis), 13 with previous history of HCC, 5 without imaging check close before therapy initiation and 2 drop-out were excluded. One hundred and fifty cases were included, among these 20 subjects with development of HCC, within a mean time of 6+4 months after stopping DAAs therapy. The cases with HCC were compared to 20 selected, among the remaining 130 cases without development of HCC, by a software for one-to-one pair-matched according to age, gender, HCV genotype and therapy schedule. All subjects gave their informed consent to participate in the study that was conducted according to the rules of Helsinki declaration and approved by the local Ethic committee protocol number N°3386/AO/14 , being part of a Regional Survey Program within HUB centers on antiviral therapy for chronic hepatitis and cirrhosis associated to HCV infection. Characteristic with the cirrhotic patients were described in **table 4.** Meanwhile, 74 healthy individuals were enrolled as a control of this study and consist of 53 males and 21 female with mean age of 56 years old (SD ± 4.2 years old).

	Study cohorts			
	Overall 40 cases	**Discovery 10 cases**	**Validation 30 cases**	**§p-value**
Age, (years±SD)	56.3±10.3	59.4±6.3	55.0±11.1	0.07
Gender male/female, (% males)	26/14 (67.5)	6/4 (70)	20/10 (66.6)	0.84
BMI, (kg/m2±SD)	25.8±3.1	27.3±2.8	25.3±3.1	0.08
Genotype HCV-1/non-1, (% HCV-1)	27/13 (67.5)	6/4 (60)	21/9 (70)	0.84
Child-Pugh score A5/A6-B7, (% A5)	30/10 (75)	8/2 (80)	22/8 (73.3)	1.0
Naive/Experienced, (% naives)	13/27 (32.5)	1/9 (10)	12/18 (40)	0.17
Sofosbuvir-based schedule, (% cases)	35/5 (87.5)	8/2 (80)	27/3 (90)	0.91
Ribavirin combination use, (% cases)	38/2 (95)	10/0 (100)	28/2 (93.3)	1.0
Treatment duration, (weeks±SD)	18.3±5.6	20.4±5.1	18.6±5.6	0.16
SVR/Relapse, (% SVR)	32/8 (80)	7/3 (70)	25/5 (83.3)	0.64
ALT (U/L±SD)	114.9±79.1	103.7±87.6	110.2±76.5	0.56
Albumin (mg/dL±SD)	40.9±3.4	41.1±3.5	39.2±3.4	0.15
Bilirubin (umol/L±SD)	15.7±7.1	14.5±6.2	16.2±8.3	0.36
PT (INR±SD)	1.1±0.08	1.0±0.05	1.1±0.09	0.76
PLTS ($x10^9$/L±SD)	164±110	120±70	179±120	0.61
Alfa-fetoprotein (ug/L±SD)	19±32	17±11	21±36	0.14
HCC development, (% cases)	20 (50)	5 (50)	15 (50)	0.71
Time to HCC diagnosis from begin of DAAs (months±SD)	8.9±5.6	9.2±5.1	8.8±5.6	0.8

Table 4: Characteristics of cirrhotic patients enrolled in study cohorts.

3.2.2. Study Design

This study was organized as follows:

1. **Discovery phase**. Serum samples from 10 patients treated with DAAs (5 pair-matched between cases with and without development of HCC) were analyzed through microarray profiling. Analysis was performed at two time points: before DAA

treatment (T0) and after 1 month of DAA treatment (T1) to explore changes among miRNA in the two groups. One-way analysis of variance (ANOVA) test was used to determine gene expression differences among subjects who developed HCC (group with HCC) *vs* cases who did not (group without HCC) **(Fig. 11).** In the discovery cohort, a panel of candidate circulating miRNAs was selected based on the differential fold of change (cut-off: ±1.5) and significance level ($p<0.05$).

2. **Validation phase.** The selected miRNA candidates were tested by quantitative Real Time PCR (RT-qPCR) in an independent cohort of 30 patients treated with DAAs (15 pair-matched between cases with and without development of HCC). Analysis was performed by estimating the time-averaged difference of a given miRNA between subjects with HCC *vs* without HCC using a bootstrapped random-effect generalized least square regression model (RE-GLS).

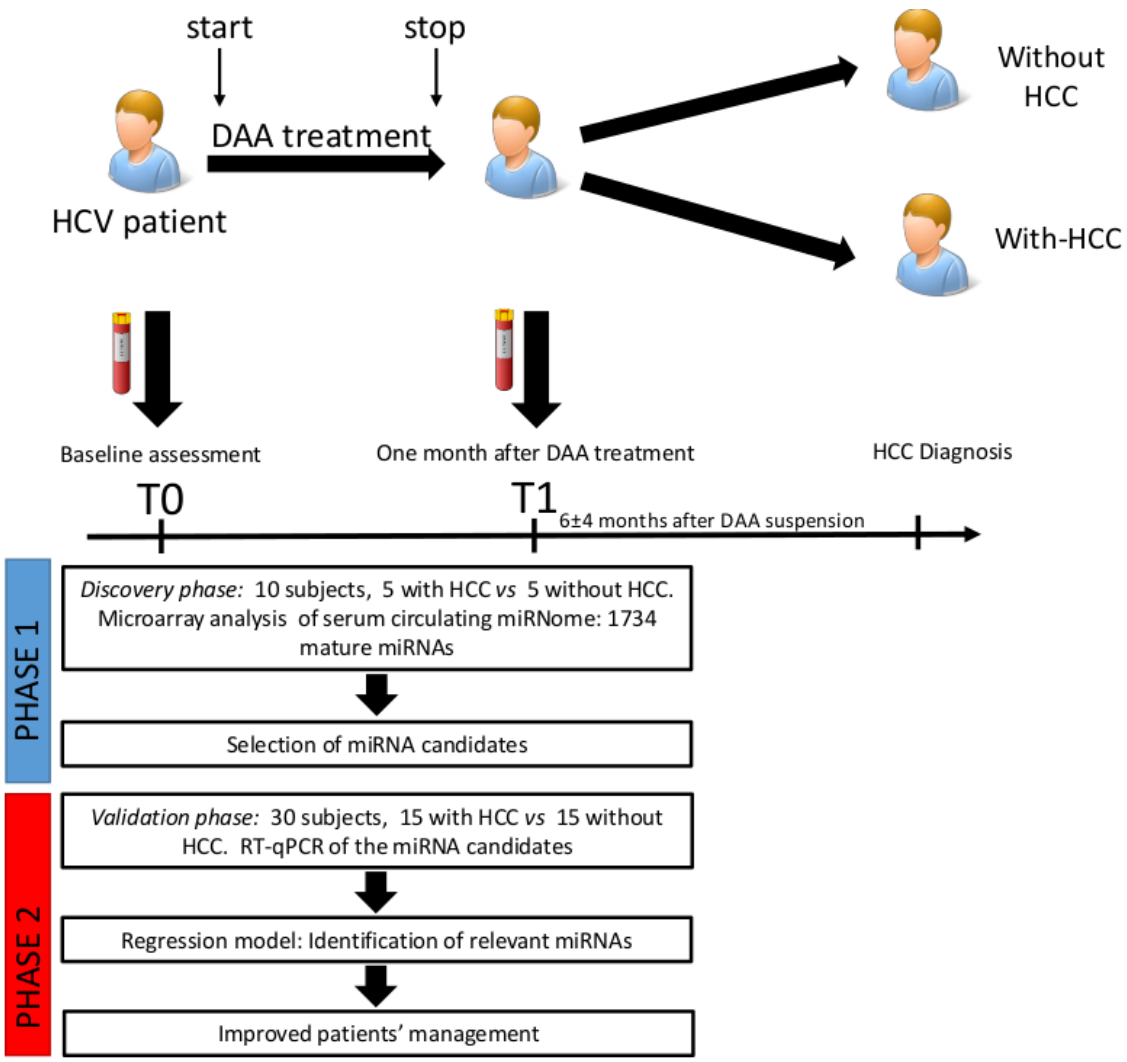


Figure 11. General scheme of the miRNA profiling study design. In the discovery phase 10 patients were enrolled and analyzed through Genechip miRNA 3.0 array. Patients were divided into two groups, HCC+ (subjects developing HCC after DAA treatment) and without-HCC- (subjects not developing HCC after DAA treatment). Samples were analyzed at T0 and T1. Subsequently miRNA candidates were assessed by qRT-PCR in a 30 patients (validation cohort). MiRNA biomarkers were selected by estimating the time-averaged difference of a given miRNA between subjects with HCC *vs* without HCC using a bootstrapped random-effect generalized least square regression model (RE-GLS).

3.2.3. Statistical Analysis

We estimated the time-averaged difference of a given miRNA between subjects with HCC *vs* without HCC using a bootstrapped random-effect generalized least square regression model (RE-GLS). The RE-GLS model used the given miRNA (continuous) as dependent variable and time (discrete; 0 = time 1; 1 = time 2), HCC (discrete; 0 = no; 1 = yes) and a time*HCC interaction (discrete*discrete) as predictors. Using such model, we used a specific contrast to estimate the time-averaged difference of the 2 repeated measures of the given miRNA in the group with HCC *vs* the group without HCC. The random effect of the RE-GLS was assigned to the patient. Internal cross-validation was performed using bootstrap on 1000 samples with replacement. This is expected to correct for over-optimism and make the model more generalizable [235]. The analysis was performed considering the ΔΔCq values. The receiver operating characteristic (ROC) curves were plotted to estimate the discriminatory potential of the miRNAs.

3.3. Task 2. Serum miRNAs as Prognostic Biomarker after HCC Treatment

3.3.1. Patient Characteristics

We conducted a real-life-practice observational study on HCC patients enrolled in Cattinara Hospital, Trieste, Italy, from January 2010 to December 2016. The major objective of the study is to identify circulating miRNA profiles that predicts prognosis of patients after treatment according to three variables:

1) therapy response (TR)

2) disease-free survival (DFS)

3) overall survival (OS)

A total of 86 cases were treated according to Barcelona Classification of Liver Cancer (BCLC) consist of surgical resection (16 patients), radiofrequency (7 patients), thermoablation (22 patients), and TACE (46 patients). All subjects gave their informed consent to participate in the study that was conducted according to the rules of Helsinki declaration and approved by the local Ethic committee (Comitato Etico Regionale Unico FVG, No. 14/2012 ASUITS). Clinical charactersitics of patients were provided in **Table 5**.

	Patients	***Curative treatment***		***TACE***		
	n=86	**CR** n=27	**PRPD** n=13	**CR** n=14	**PDPR** n=32	***P value***
Age (mean, 95%CI)	72.4 (70.8-74.0)	71.8 (69.1-74.5)	74.3 (69.4-79.1)	74.4 (68.8-80)	69.6 (66.7-72.5)	ns
Sex (M/F)	69/17	21/6	10/3	8/6	30/2	*P<0.01*
Etiology						
Alcohol metabolic	46	12	7	7	20	*ns*
Alcohol metabolic viral	14	5	3	3	3	
Viral	20	7	3	3	7	
Other*	5	2	0	1	2	
Disease scores						
CTP A/B	64/19	20/6	12/1	12/2	20/10	*ns*
BCLC 0A/BC	61/24	26/1	12/1	9/5	14/17	*P<0.001*
Number of lesions						
Single < 2cm	10	4	2	2	2	*ns*
Single or 3 ≤3cm	45	19	8	6	12	
Large-single or multi	30	4	3	6	17	

Alpha fetoprotein						
<20 ng/mL	49	16	7	10	16	*ns*
20 - 400 ng/mL	12	2	3	1	6	
>400 ng/mL	6	2	1		3	

Table 5: Characteristics of HCC patients enrolled in study cohorts.

3.3.2. Study Design

This study was organized as follows:

1. **Discovery phase**. Serum samples from 22 HCC patients were analyzed through microarray profiling. Analysis was performed at three time points: before HCC treatment (T0), after 1 month of treatment (T1) and after 6 months of treatment (T6) to explore changes among miRNA before and after treatment **(Fig. 12).** One-way analysis of variance (ANOVA) test was used to determine miRNA expression differences among subjects responding to treatment (responder) and not responding to treatment (non-responder) according to TR, DFS, and OS. Multiple testing correction was performed with the Benjamini-Hochberg method. In the discovery cohort, a panel of candidate circulating miRNAs was selected based on the differential fold of change (cut-off: ±1.5) and significance level ($p<0.05$).
2. **Validation phase.** The selected miRNA candidates were tested by quantitative Real Time PCR (RT-qPCR) in an independent cohort of 86 HCC in the same time points as the discovery phase. Analysis was performed by distinguishing the patients into responder and non-responder. The receiver operating characteristic (ROC) curves were plotted to estimate the discriminatory potential of the miRNAs. Kaplan-Meier survival analysis was used to estimate the potential of significant miRNAs to predict overall survival after treatment.

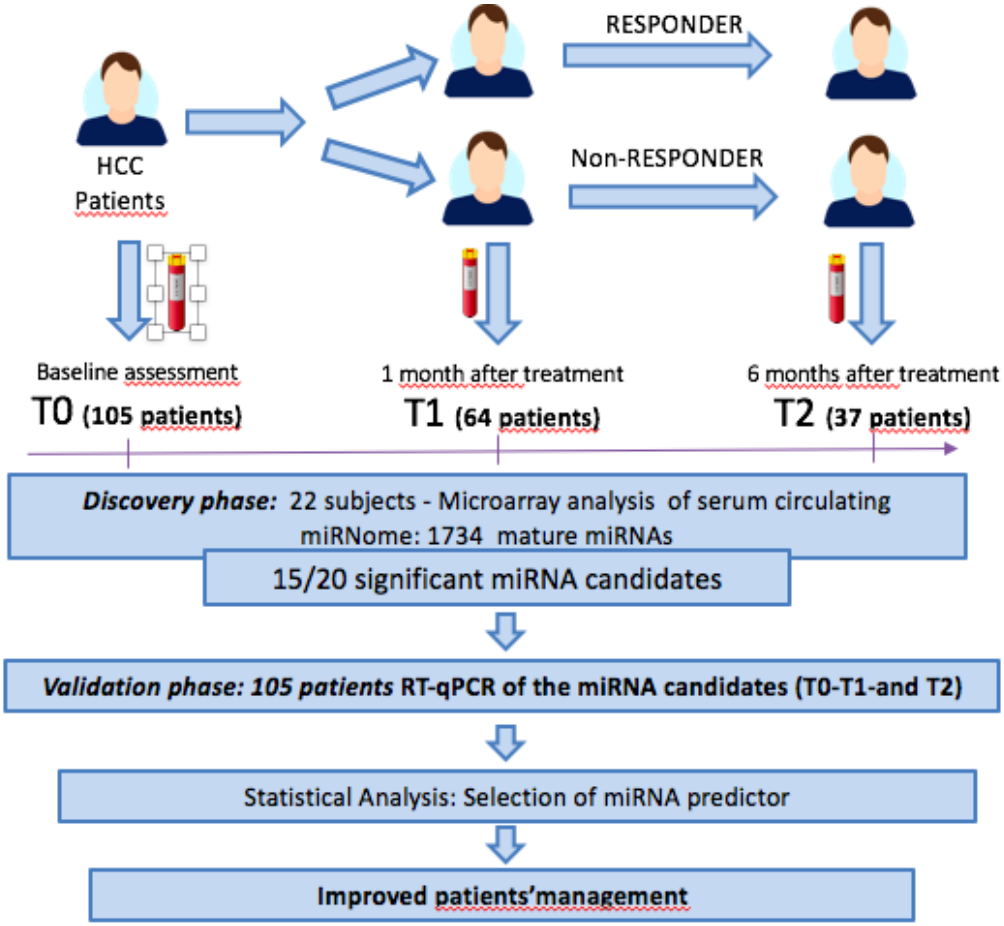


Figure 12. General scheme of the miRNA profiling study design for Task 2. In the discovery phase 10 patients were enrolled and analyzed through Genechip miRNA 3.0 array. Patients were followed and divided based on their response to therapy. Samples were analyzed at T0 (before therapy), T1 (one month after therapy), and T6 (six months after therapy). Subsequently miRNA candidates were assessed by qRT-PCR in 86 patients (validation cohort).

3.4. Task 3 - Validation of miRNA Targets and Cellular Pathway

3.4.1. *In Silico* Gene Prediction Analysis

The *in silico* analysis to predict gene targets of our miRNA of interest underwent three phases consist of:

a) **Prediction analysis from miRNA databases**

Three different miRNA databases were used in this analysis. TargetScanHuman 7.2 (http://www.targetscan.org/vert_72/) predicts biological targets of miRNAs by

searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA, as well as the poorly conserved sites [236]. Predictions are ranked based on their predicted efficacy of targeting as calculated using cumulative weighted context++ scores of the site [236]. MiRDB (http://www.mirdb.org) predicted the target by analyzing thousands of miRNA-target interactions from high throughput sequencing experiments [237]. For mirDB, predicted target were ranked according to associated PubMed records for each miRNA, level of conversation in species, and expression score based on normalized read counts from 81 RNA-seq experiments. DIANA TOOLS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) used the positive and negative set of miRNA recognition elements (MREs) located in both the 3'UTR and CDS regions [238,239].

b) Cumulative ranks of shared targets from miRNA databases

All the predicted targets from the three databases were collected and combined using *Venn's* Diagram to identifie the shared targets. Shared target genes from at least two databases were chosen for further analysis. Genes were then ranked according to the mean of their respective ranking in all the databases using the following formula: Overall rank of Gene A = (TargetScan rank +miRDB rank +DIANA TOOLS rank)/3.

c) Gene characterization and target selection

The information available about the putative target gene was searched in Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/) and GeneCards (https://www.genecards.org) [240]. Genes were filtered based on their role in cancer. Genes having a proven role in any cancer pathway were considered for further *in vitro* validation experiments. Gene that produce secreted proteins obtained a priority in the selection.

3.4.2. Validation in Human Tissue

Ten paired tumoral and tumor-adjacent liver tissue samples from HCC patients were obtained from Cattinara Hospital, Trieste, Italy. All subjects gave their informed consent to participate in the study that was conducted according to the rules of Helsinki declaration and approved by the local Ethic committee. All specimens were collected within 15 minutes after

removal and were immediately snap-frozen in liquid nitrogen before storage at -80 C. Tissue samples were homogenized using 1 ml of TRI-reagent per 50-100 mg of tissue using a glass-teflon of homogenizer. RNA was extracted as previously described. Paired tissues were used to investigate the miRNA and targets expression.

	N=10
Gender (M/F)	8/2
Age (Mean, SD)	68,932 (± 9,09)
Treatment (Resection/Thermoablation)	9/1
Overall Survival (months, mean, SD)	33.71 (± 30,38)
Overall Survival (≤24/24 months)	4/5
Disease Free Survival (months, mean, SD)	24,31 (± 25,7)
Disease Free Survival (≤24/24 months)	2/6
Therapy Response (Complete/Non-Responder)	7/3

Table 6: Characteristics of HCC patients enrolled in study cohorts.

Task 3.4.3. *In Vitro* miRNA pathway analysis

3.4.3.1. Cell lines and culture conditions

JHH6 (undifferentiated human hepatocellular carcinoma) were obtained from Japan Health Science Research Resources Bank (HSRRB, JCRB1030) and were cultured in Williams E medium with 10% (v/v) FBS, 1% L-glutamine 100X, 1% penicillin/streptomycin 100X. JHH6 were grown as monolayer culture at 37°C in 5% CO_2 and 95% humidity.

3.4.3.2. Total protein extraction

Cells were washed twice with cold PBS and than lysed with 200µL of Cell Lysis Buffer (10X) and maintained on ice for 5 min. The cells were then scraped and sonicated briefly (3 pulses of 5s at 10W) using a sonicator UW3100 (Bandelin electronics, Berlin). The extracts were centrifuged at 14000 x g for 10 min at 4°C. The supernatant was collected for protein quantification by reaction with Bicinchoninic Acid Solution-KIT according to manufacturer's instructions.

3.4.3.3. MTT assay

The cell viability in terms of mitochondrial activity was determined by the MTT assay

[362,363]. Typically, the cells were cultured in a 6 multiwell plate. After the treatment the cell culture medium was removed the wells were washed with a 9.6g/L solution of PBS pH 7.2-7.6. 50µL of a MTT stock dye solution (5mg/ml in PBS) was added to each well containing 450µL of fresh medium. The plates were incubated at 37 °C under 5% CO2 for 1h. The medium from each well was removed and 500µL of DMSO (Sigma-Aldrich D5879-L) were added to dissolve the purple formazan crystals. The plates were shaken for 10min and the absorbance for each well was read on microplate reader (Beckman Coulter LD 400C Luminescence detector) at 570nm. The fractional absorbance was calculated by the following formula: % Cell survival = (mean absorbance in test well)/(mean absorbance in control wells) * 100.

3.4.3.4. SDS-page Western Blot analysis

Total proteins (20µg for JHH6) were solubilised in Laemmli Buffer 5X and 10% β-mercaptoethanol, separated with 10% SDS/PAGE gel and transferred to nitrocellulose membranes by electroblotting, using 25mM Tris Base, 192mM glycine, 20% methanol as transfer solution. After the transfer, the membranes were blocked for 1h in 4% bovine serum albumin in TTBS (100mMTris Base, 2.5M NaCl, TWEEN 20 1% pH 7.5). Subsequently the membranes were incubated overnight at 4°C with the respective primary antibodies in blocking solution at 1:500 dilution for Monoclonal Anti-DLG5 antibody (Sigma-Aldrich, St. Louis, MS, USA) and 1:2000 for Actin (Sigma-Aldrich, St. Louis, MS, USA). After washing 3 times for 10 min in blocking solution, immune-complexes were detected with the respective secondary antibodies of the predicted gene target after 60 min incubation. Later, membranes were washed (3 x 5 min Blocking solution, 1 x 5 min T-TBS 1 x 5 min TBS) and the bands were visualizated using Luminata™ Western HRP substrate by following the manufacturer's protocol.

3.4.3.5. MiRNA Transfection with mimics

Mir-4454 mimic is purchased by Riboxx Pharmaceuticals (Radebeul, Germany). This mimic is a double-stranded RNAs designed by the manufacturer able to be load into RISC complex and mimic the function of their specific miRNAs. MiRNA mimic (20 nmol) were diluted in 500 ul of RNAse free water to reach the stock concentration of 2uM/ul, to be stored in -20°C. A double-stranded scramble miRNA from *Caenorhabditis elegans* designed by MISSION™ synthetic were purchased from Sigma-Aldrich (St. Louis, MS, USA) as negative

control. Transfection efficiencies and working concentrations of the miR inhibitors were determined during set-up experiments. SilentFect™ lipid transfection reagent (Biorad, California, USA) was used as a delivery system for the transfection. The transfection protocol was determined according to ratios surface area/RNA/SilentFect given by the manufacturer **(Table 7).** Transfection efficiency was determined by FACS analysis using a FITCH conjugated miRNA-, as 99.4% and 90.8% of efficiency for 6000 cells/cm^2 and 8000 cells/cm^2, respectively.

Culture Vessel Size	Volume of Plating Media	siRNA conc.	Volume of Serum Free Medium	siLentFect Reagent
96 well	0.1 ml	5–20 nM	20 µl	0.05–0.4 µl
24 well	0.5 ml*	5–20 nM	50 µl	0.25–2.0 µl
12 well	1.0 ml*	5–20 nM	100 µl	0.5–4.0 µl
6 well/35mm	2.5 ml*	5–20 nM	250 µl	1.0–5.0 µl
60 mm	5.0 ml*	5–20 nM	500 µl	2.5–10 µl
100 mm	10.0 ml*	5–20 nM	1.0 ml	5.0–20 µl

Table 7. Transfection protocol for siLentFect™ transfection reagent (Bio-Rad, CA, USA).

3.4.3.6. Transfection Experiment

JHH6 cells (8000 cells/cm^3) were seeded in six-well plate and incubated overnight at 37° C in humidified atmosphere with 5% of CO_2. The following day, an hour before treatment, cells are washed with PBS and incubated in 2 ml of fresh medium. Cells were then transfected with the mixture of serum-free medium, siLentFect™, and miRNA mimic (concentration: 25nM and 50 nM) for 48 hours. 50nM of the negative control (*C. elegans derived miR*) and cells without treatment were included into the experiment. Fresh medium was added at 24 hours post transfection. RNA and proteins were then extracted as mentioned before. Three replicates were conducted for this experiment.

3.4.3.7. Cell cycle FACS analysis

After 72h, transfected cells were detached, pelleted, washed twice with PBS and then resuspended in 500µL PBS. Cells were homogenized in order to disaggregate the eventual groups of cells for obtaining a single cell suspension, subsequently they were transferred to a fresh tube containing 4.5mL of ethanol 70%. Fixed cells were pelleted, to remove the ethanol,

and resuspended in 1mL of staining solution (0.1% v/v TritonX-100 in PBS; 0.02 mg/mL PI; 0.2 mg/mL RNAse A). After 30 min of RT incubation, cellular DNA content was measured by flow cytometry using a Becton Dickinson FACSCalibur System, following excitation with an argon ion laser source at 488nm and appropriate filter sets. Data were collected in 10,000 cells and analyzed using Cellquest software from BD Biosciences (San Jose, CA, USA). The percentage of cells in G0/G1, S and G2/M was determined from DNA content histograms.

3.4.3.8. Analysis of cell apoptosis

8000 cells/cm^2 were seeded in 6-well plate overnight. Cells were then treated with miRNA inhibitor/mimic and were prepared for flowcytometry analysis after 48 or 72 hours incubation time. Cells were then detached, pelleted, washed with PBS, then resuspended in 500 ul of binding buffer (10mM Hepes/NAOH, pH 7.40, 140mM NaCl, 5 mM $CaCl_2$). Suspension of Cells were incubated for 15 minutes in room temperatue. Cells were stained with5ul of PI and 5 ul of Annexin added to 100ul of cell's suspension and incubated for 10 minutes. Flow cytometery analysis was performed using Becton Dickinson FACSCalibur System to a population of 10.000 cells. Campothecin- treated cells (Campothecin concentration: 25 uM) were used as positive control.

3.4.3.9. Wound scratch assay

The abilites of miRNAs to influence cell migration and invasion were assessed by wound scratch assay. The transfected cells (8000 cells/cm^2) in were seeded in 24-wells plate and allowed to proliferate until 90% of confluence was achieved. After 24 hours post transfection, the culture medium was aspirated and cells are washed with PBS. Wounds were created with a diagonal scratches drawn on the surface of the monolayer cells with a 20ul pipette tip. Debris was removed through a PBS rinse, and the distance between the wound edges were measured using the microscope. Cells were then left to be incubated for another 24 hours. The distance between the wound edges were then measured again using the microscope, and the changes of distance between edges were photographed in 10x magnification and reported. Two replications were performed for this experiment.

Chapter 4

Results

4.1. TASK 1 - Serum miRNA biomarkers for early hepatocellular carcinoma occurrence following direct-acting antivirals treatment

4.1.1. MicroRNA screening in the discovery cohort, identification of predictive miRNA

Based on the previous result from microarray profiling analysis, 9 miRNAs, consist of miR-1207-5p, miR-1275, miR-3197, miR-4443, miR-3178, miR-483-5p, miR-4706, miR-4793-3p and miR-1246, were significantly and differently expressed between the group with HCC (HCC+) and without HCC (HCC-) at T0 (Anova $p<0.05$) **(Fig. 13)**. A different miRNA profile distinguishing the two groups, was identified at T1 including miR-1180-3p, miR-1228, miR-4329 and miR-4484 **(Fig. 13).**

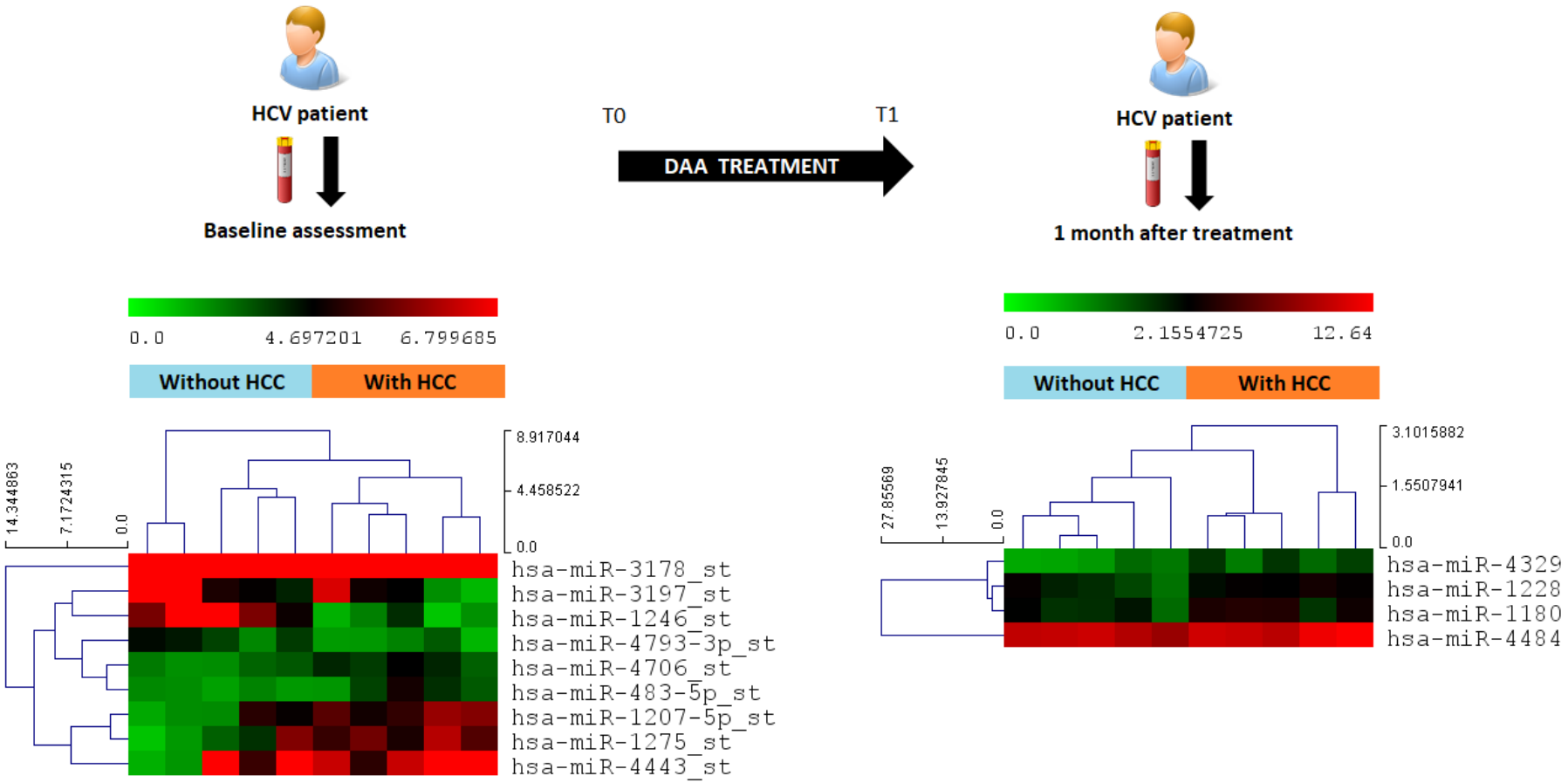


Figure 13. Serum samples were collected from patients before and after DAA treatment initiation. Circulating miRNome profiles were analyzed by miRNA array at both times. Statistically significat miRNAs were included into the heatmap with the pseudocolor scale underneath. Unsupervised hierarchical clustering is used to order samples and miRNAs, the log2-transformed microarray signal was considered. The sample tree with optimized leaf-ordering is drawn using Euclidean distances and average linkages for cluster-to-cluster distance.

4.1.2. Differential miRNA expression profiles and identification of miRNA biomarker candidates

We used the RT-qPCR assays to confirm the expression of 13 miRNAs candidates that were differently expressed ($p<0.05$) in the discovery cohort at T0 and T1. We assessed the miRNA expression in further 60 samples, in which half of them developed tumor after the DAA therapy. Among the selected miRNAs, we were not able to confirm through RT-qPCR assays the expression of miR-4706, mir-4793-3p, and miR-4329. In addition, the variance among samples of miR-1275 was extremely low (0.35) compared to the other samples that was discarded for subsequent analysis. MiRNA expression data are reported in **Table 8.**

miRNA	With HCC mean ΔΔCq (95%CI)	Without HCC meanΔΔCq (95%CI)
miR-483-5p	1.04 (0.62-1.47)	0.80 (0.51-1.10)
miR-1246	0.48 (0.34-0.63)	0.42 (0.24-0.61)
miR-1180-3p	0.37 (0.13-0.62)	0.18 (0.12-0.24)
miR-3197	0.33 (0.14-0.52)	0.80 (0.48-1.12)
miR-4443	0.14 (0.04-0.24)	0.10 (0.07-0.14)
miR-3178	2.61 (2.12-3.12	2.01 (1.48-2.53)
miR-1207-5p	0.62 (0.46-0.79)	0.81 (0.51-1.12)
miR-1228-3p	0.71 (0.42-0.99)	0.97 (0.71-1.2)
miR-4484	0.66 (0.43-0.88)	0.42 (0.33-0.51)

Table 8. Expression levels of the miRNA candidates in the two groups

When estimating the time-averaged difference of the two-repeated measures for each miRNA between HCC+ and HCC-, we identified significant changes, considering both time and disease for miR-3197, showing a lower expression in HCC **(Fig. 14g).** Interestingly, miR-3197 showed the highest differences in the expression between the two considered groups.

Despite not being statistically significant, miR-483-5p, miR-1246, miR-3178, miR-1207-5p and miR-4484 shown a higher expression in patients with HCC. While miR-1180-3p and miR-4443 had no differences both in terms of time and presence of the disease **(Fig. 14b and 14c)**.

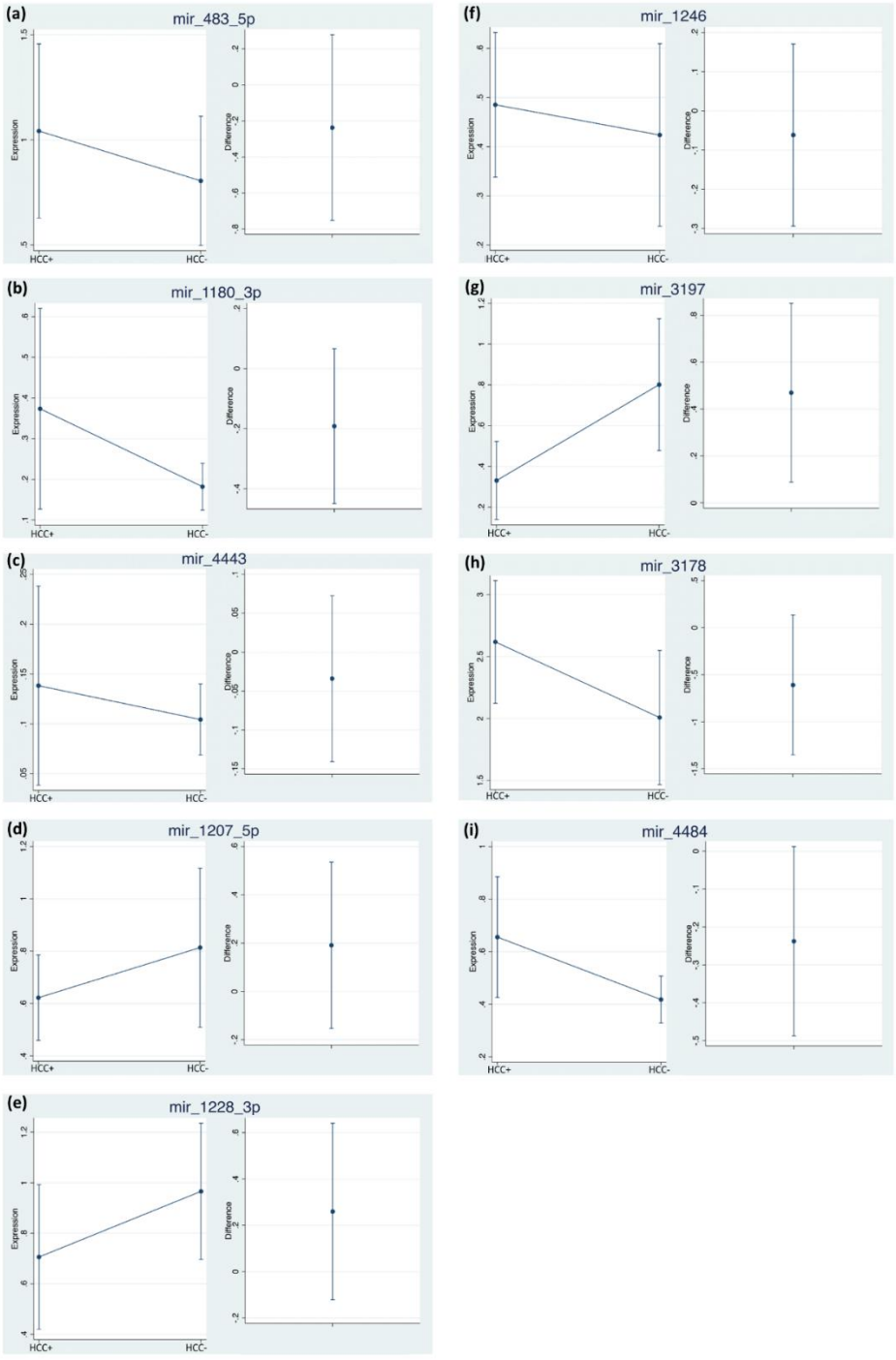


Figure 14. Time-averaged differences of miRNAs between subjects with HCC (HCC+) vs. those without HCC (HCC-). The estimates were obtained using bootstrapped random-effect generalized least square regression (RE-GLS) calculated on the Cq expression values. Internal cross-validation was performed using bootstrap analysis on 1000 samples with replacement. (See statistical analysis for details). The Difference is calculated as (HCC+ minus HCC-), values shoes 95%CI cross = are not statistically significant.

4.1.3. MiR-3197 is differently expressed between healthy iondividuals and cirrhotic patients that develop HCC

In order to validate the diagnostic potential of miR-3197, We decided to perform an explorative study comparing the expression of miR-3197 between HCC+, HCC- and 74 healthy individuals by qRT-PCR analysis. Using non-parametric Kruskal-Wallis in one way ANOVA procedure test, we calculated the mean ΔΔCq expression of miR-3197 of the three groups: the mean ΔΔCq was 0.16 (0.12-0.19) for healthy individuals, 0.13 (0.09-0.22) for HCC-, and 0.06 (0.03-0.09) for HCC(+). The ΔΔCq mean of miR-3197 in HCC+ patients was significantly reduced compared to both healthy individuals (p= 0.008), being 2.66 times lower, and HCC- (p= 0.027) being 2.17 lower. There were no statistically significant differences between healthy individuals and HCC- cirrhotic(p= 0.959) (**Fig. 15a).**

In order to validate the diagnostic potential of miR-3197, we calculated the area under the curve (AUC) of the ROC curve for miR-3197. Using a cut-off of ΔΔCq ≤ 0.12, miR-3197 **(Fig. 15b)** can distinguish all the cirrhotic patients) from healthy individuals with a sensitivity and specificity of 76.5% and 53.5%, respectively (AUC= 0.66 (0.53-0.76, 95% CI), p= 0.003). Using a cut-off of ΔΔCq ≤ 0.07, miR-3197 **(Fig. 15c)** can distinguish HCC+ from healthy individuals with a sensitivity and specificity of 68.4% and 67.6%, respectively (AUC= 0.75 (95% CI 0.60-0.85), p< 0.001). Furthermore, using a cut-off of ΔΔCq ≤ 0.08, miR-3197 **(Fig. 15d)** can distinguish HCC+ patients from HCC- patients with a sensitivity and specificity of 79.0% and 70.0%, respectively (AUC= 0.78 (95%CI 0.58-0.89), p< 0.001).

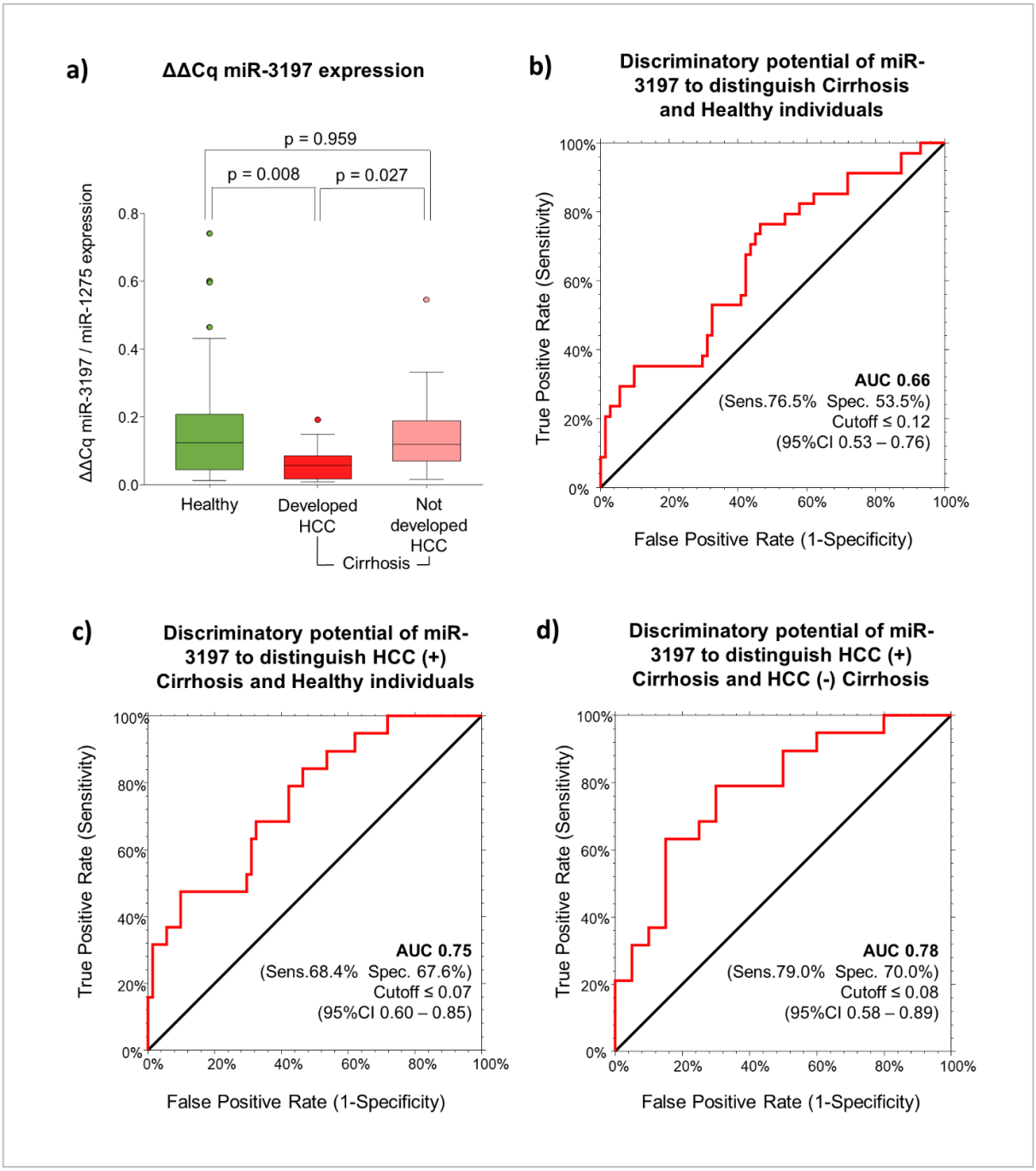


Figure 15. analyses on miR-3197 between HCC+ patients, HCC- patients, and healthy individuals. Mean ΔΔCq expression of miR-3197 in the three groups (**a**). ROC Curve analyses which show the discriminatory potential of miR-3197 between cirrhosis and healthy individuals (**b**), HCC+ and healthy individuals (**c**), and HCC+ and HCC- cirrhosis (**d**).

4.2. TASK 2 - Serum miRNAs as Prognostic Biomarker after HCC Treatments

4.2.1. MicroRNA screening in the discovery cohort, identification of predictive miRNA

Based on the previous results from microarray profiling, 9 miRNAs consisting of miR-1246, miR-3185, miR-4492, miR-4454, miR-4530, miR-4443-5p, miR-4423-3p, miR-4507 and miR-335-3p were significantly associated with prognostic variables. Mir-4454, miR-4492, miR-4443 and miR-4530 were significantly associated with therapy response (TR). MiR-4423, miR-3185, miR-4507, and miR-335-3p were associated with overall survival (OS). Mir-1246, miR-4454 and miR-4530 were associated with disease-free survival (DFS). Then, we used the RT-qPCR assays to confirm the expression of 9 miRNAs candidates that were differently expressed ($p<0.05$) in the discovery cohort at T0, T1, and T6 in 105 samples. The mean ΔΔCq expression for all miRNAs are summarized in **Table 9, 10, and 11.**

4.2.2. Panel of Serum miRNAs Associated with Therapy Response

Patients were divided into Complete Responders (CR), Partial Responders (PR) and non-responders/progressive disease (NR) according to *mRECIST criteria* [241]. CR patients were defined as disappearance of all target and non-target nodes to <10mm in the short axis from radiological imaginG, PR were defined as ≥ 30% decrease in tumor burden compared to the largest diameter in axial plane of tumor, while non-responders were defined as neither partial, complete response, and include disease progression (increase of tumor burden or appearance of new lesions) [242]. PR and NR patients were grouped together and named PR. Four miRNAs, miR-3185, miR-4492, miR-4454 and miR-4530 were significantly and differently expressed between CR and PR at T0 **(Table 9)**, showing an increased fold of change of 1.40, 1.84, 1.88, and 1.42 in CR, respectively. Meanwhile at T1, none of the miRNAs had significant differences, even though we observed 2 times increase in CR for miR-4454. At T6, miR-4454 was significantly upregulated in CR, 4.92 times higher compared to PR ($p= 0.01$) **(Table 9).** In order to better clarify the prognostic significance for the miRNA candidates, we considered separately the groups of patients receiving either curative treatments (liver resection and radiofrequency) or TACE.

4.2.3. Panel of Serum miRNAs Associated with Therapy Response for curative treatments

Considering the only the patients treated with curative therapies (n=41), three miRNAs consisting of miR-4454, miR-4443 and miR-4530 were confirmed as significantly and differently expressed between CR and PR at T0. The expression of miR-4454 was higher in CR at all considered times point, however being significant only at T0 (p=0.02) **(Fig. 16a).** Considering the overall variation, miR-4454 is stably expressed at low levels in PR from T0 to T6 while it is always higher in CR, reaching the highest difference with the PR group at T6 (**Fig. 16j).** The expression of miR-4530 was significantly higher in CR only at T0 (p=0.008) (**Fig. 16c)**, even though it is shown that CR had a relatively high expression of miR-4530 from T0 to T6 in CR compared to PR (p=0.04) **(Fig. 16l).** Conversely, miR-4443 was significantly downregulated in CR compared to PR at T0 (p=0.05) **(Fig. 16b)**, but not signifcant at T1 and T6 **(Fig. 16e, 16h).** MiR-4492 was associated to therapy response in the discovery phase, and was not confirmed as significant in curative treatments.

To validate the discriminatory potential of the three miRNAs, the area under the curve (AUC) was determined with a ROC analysis. Due to the importance to identify and predict responder patients before HCC treatment, the analysis was performed at T0 for each miRNAs. The highest AUC values were obtained for miR-4530 (AUC=0.77, 0.55-0.89, 95%CI), with a sensitivity and specificity of 72% and 72.2%, respectivelly, at a cut off determined at 31.74. The corresponding AUC values for miR-4454 and miR-4443 were 0.74 (95% CI 0.49-0.88) and 0.72 (95% CI 0.45-0.87), respectively **(Fig. 17).** Mir-4454 has the highest sensitivity in distinguishing CR from PR before therapy (sens= 79%). The combination of the three miRNA showed a greater ability than any individual miRNA to distinguish CR from PR with an AUC of 0.84 (95% CI 0.45-0.87) using an optimal cut-off value of 0.50, which demonstrated a sensitivity of 72% and specificity of 75% (logit model formula: -1.89 + 0.32*miR-4492 + 6.45*miR-4454 – 0.01*miR-4492*miR-4492 – 0.75*miR-4492*miR-4454 – 0.90*miR-4454*miR-4454) **(Fig. 17d).** Patients were then divided into Low *vs* High miR expression using the mean expression value of PR as a cutoff. Kaplan-Meier survival analysis demonstrated that high expression of the miR4454 was significantly related to better overall survival in patients before initiation of therapy (T0) with HR of 2.63 (95% CI 0.15-0.93, p=0.016) **(Fig 18a).**

	T0			T1			T6		
miRNA	**CR (95% CI)**	**PR (95% CI)**	**P value**	**CR (95% CI)**	**PR (95% CI)**	**P value**	**CR (95% CI)**	**PR (95% CI)**	**P value**
miR-1246	5.32 (3.48-7.16)	5.05 (3.02-7.07)	0.39	20.47 (10.85-30.09)	23.12 (11.26-34.99)	0.56	14.32 (1.36-27.28)	5.35 (2.57-8.14)	0.30
miR-3185	36.08 (28.12-44.03)	25.69 (20.82-30.56)	**0.037***						
miR-4492	21.86 (15.55-28.16)	11.84 (9.10-14.57)	**0.006*****	15.80 (9.98-21.61)	12.66 (8.64-16.70)	0.61			
miR-4454	0.32 (0.22-0.43)	0.17 (0.11-0.23)	**0.01****	0.40 (0.13-0.66)	0.80 (0.48-1.12)	0.33	0.64 (0.34-0.95)	0.13 (0.06-0.20)	**0.01****
miR-4530	79.19 (52.60-105.7)	55.54 (28.94-82.1)	**0.015***	77.29(47.18-107.39)	81.60 (54.65-108.5)	0.54	85.05 (48.27-121.84)	86.49 (45.41-127.57)	0.51
miR-4443	2.7(1.86-3.54)	5.14 (3.21-7.07)	0.089	6.93 (3.89-9.97)	8.79 (3.76-13.83)	0.67	6.55 (1.15-11.94)	5.93 (3.69-8.16)	0.14
miR-4507	34.50 (21.44-47.55)	19.12 (6.52-31.73)	0.084						
miR-335-3p	0.08 (0.037-0.12)	0.11 (0.035-0.19)	0.85						
miR-4423-3p	0.29 (0.21-0.38)	0.31 (0.24-0.40)	0.60						

Table 9. Expression levels of the miRNA candidates based on response to therapy at T0, T1 and T6.

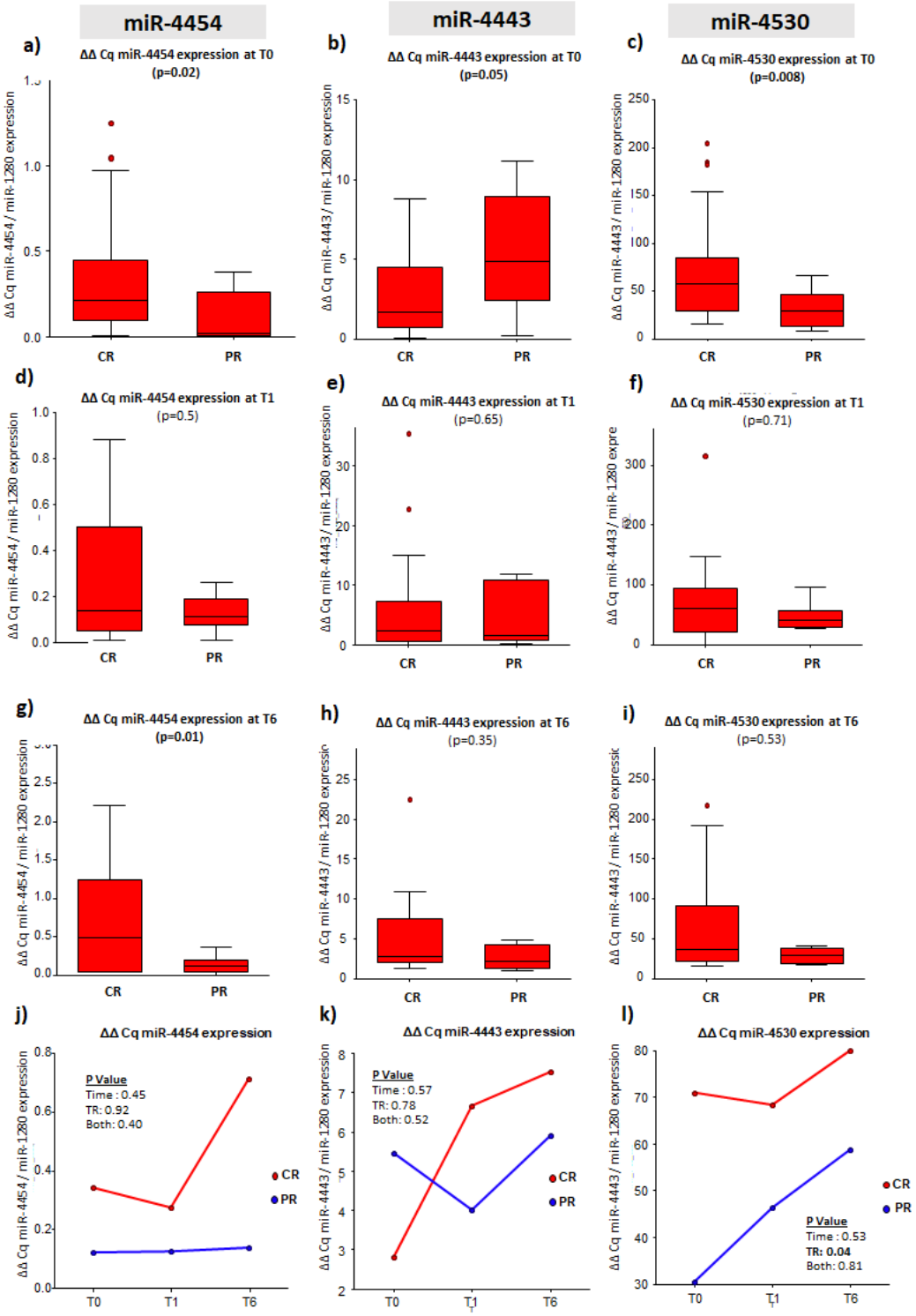


Figure 16. Mean ΔΔCq expression of miR-4454, miR-4443 and miR-4530 in CR and PR treated with curative therapies.

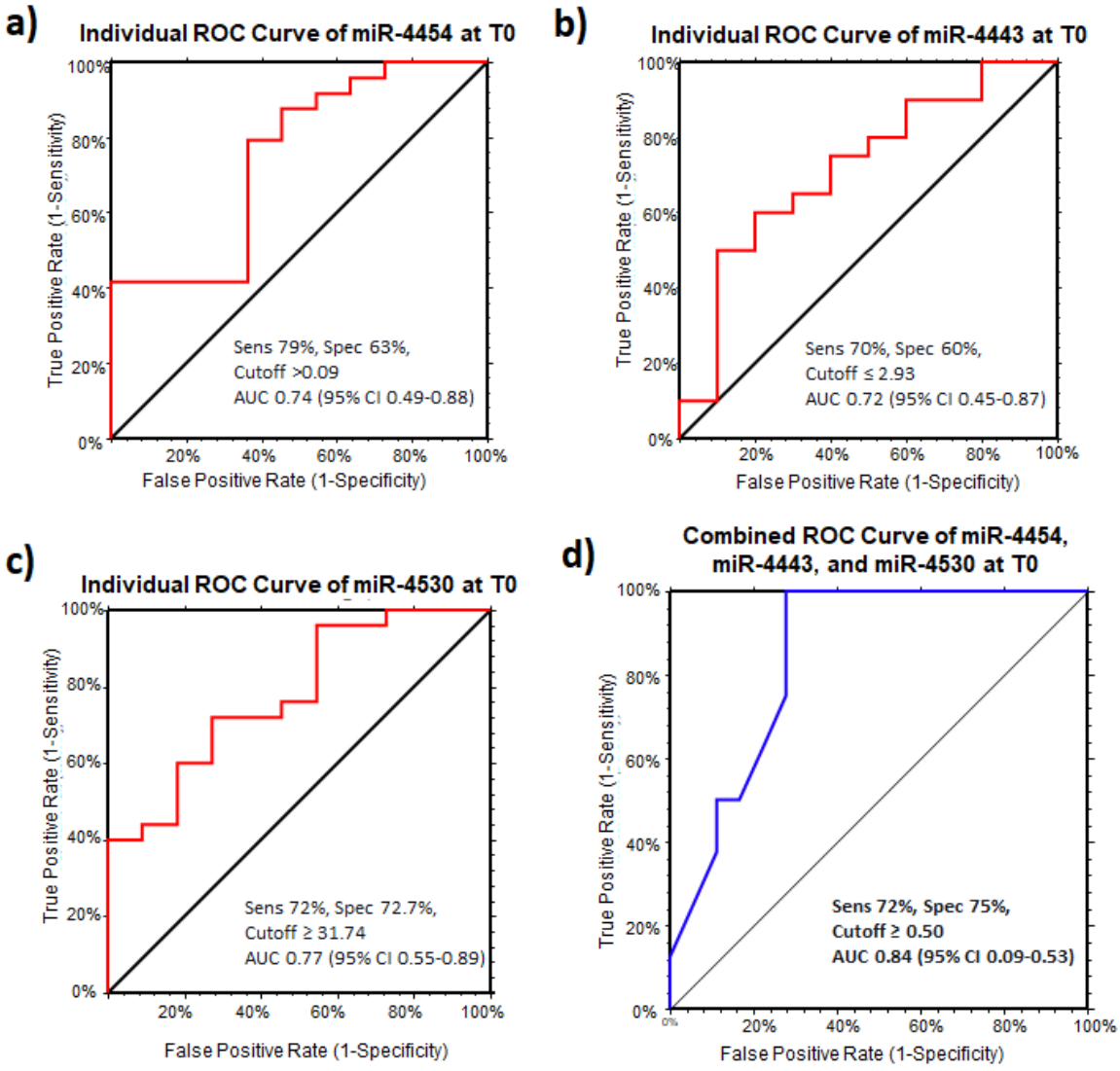


Figure 17. Receiver operating Curve (ROC) analysis of miR-4454 (a), miR-4443 (b), miR-4530 (C), and combination of the three miRNAs (d) to determine the accuracy on distinguishing complete responder (CR) from partial responder (PR) before curative therapy (T0).

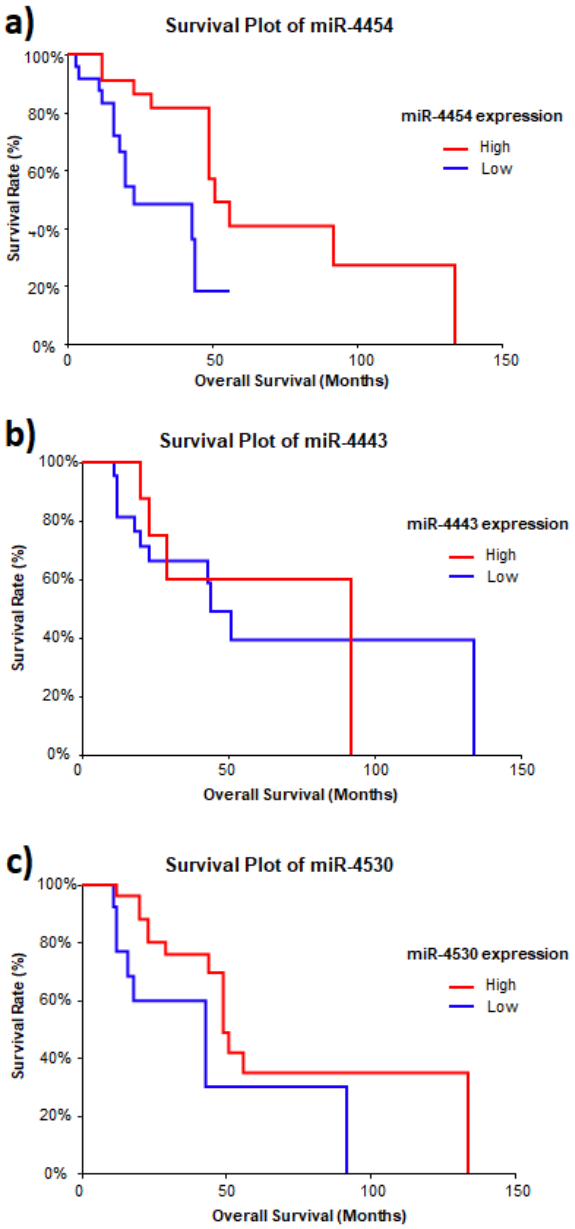


Figure 18. Kaplan-meier survival analysis by log-rank test between high and low expression of miR-4454 (a), miR-4443 (b), and miR-4530 (c) at T0 for patients receiving curative therapies.

4.2.4. Serum miRNAs Associated with Therapy Response in TACE

We analyzed our miRNA candidates in HCC patients receiving trans-arterial chemoembolization (TACE) as a non-curative treatment. None of miRNAs, able to significantly distinguish CR and PR in curative treatment, were found to be statistically and differently expressed between the two populations in TACE **(Fig. 19)**, even though we noticed a pattern of upregulation for miR-4454 in CR at T0, T1 and T6 (**Fig. 19a, 19d, 19g).** In the opposite, miR-

4492 was differently express between CR and PR in curative therapies **(Fig. 20a, 20c)**, but significantly upregulated for CR to TACE at T0 **(Fig. 20b).** Mir-4492 shown a trend of upregulation in CR from T0 to T1 compared to PR (p=0.03) (**Fig. 20f).** Despite the great difference at T0, miR-4492 expression in CR reached comparable levels to PR after therapy **(Fig. 20f).** The discriminatory potential of miR-4492 was measured by calculating the area under the curve (AUC) in a ROC curve analysis for patients treated with TACE at T0. The corresponding AUC value was 0.84 (95% CI 0.57-0.91) for differentiating CR patients compared to PR with sensitivity and specificity of 84.6% and 71%, respectively, using a cut-off of 12.60 **(Fig. 20h).**

	T0				T1				T6			
miRNA	**<12 months**	**12-24 months**	**>24 months**	**P value**	**<12 months**	**12-24 months**	**>24 months**	**P value**	**<12 months**	**12-24 months**	**>24 months**	**P value**
miR-1246	5.39 (3.47-11.54)	5.06 (2.95-7.18)	4.58 (2.69-6.47)	0.25	10.15 (0.04-23.25)	18.17 (7.23-29.10)	26.60 (14.39-38.81)	0.30	4.74 (5.2-61.49)	8.63 (1.6-18.90)	12.36 (0.63-24.08)	0.30
miR-3185	20.90 (16.41-25.40)	29.34 (21.56-37.13)	35.59 (28.65-42.53)	**0.0014***								
miR-4492	19.24 (0.74-37.74)	14.22 (9.04-19.39)	19.24(13.88-24.82)	0.62	10.72 (4.42-17.00)	13.58 (7.98-19.17)	15.49 (9.64-21.34)	0.63				
miR-4454	0.16 (0.09-0.23)	0.20 (0.11-0.29)	0.28 (0.19-0.38)	0.15	0.52 (0.25-1.28)	0.24 (0.10-0.39)	0.25 (0.12-0.39)	0.36	0.17 (1.94-2.29)	0.33 (0.03-0.63)	0.52 (0.23-0.81)	0.60
miR-4530	54.95 (20.24-89.66)	64.72 (26.35-103.09)	74.61 (49.9-0.2-99.42)	0.68	97.50 (11.57-183.41)	95.6 (52.27-139.04)	64.08 (47.62-80.55)	0.26	52.16 (30.89-413.24)	112.4 (60.0-164.85)	75.74 (42.14-109.34)	0.51
miR-4443	5.42(2.02-8.83)	3.85 (2.52-5.18)	3.09 (2.09-4.08)	0.16	5.40 (1.20-9.60)	9.62 (2.75-16.50)	7.29 (1.82-11.01)	0.60	5.08 (2.06-6.20)	4.92 (2.55-7.30)	7.11 (1.74-12.49)	0.14
miR-4507	1.26 (0.53-3.06)	28.21 (9.75-46.67)	31.51 (20.21-42.82)	**<0.001****								
miR-335-3p	0.14 (0.011-0.27)	0.09 (0.035-0.19)	0.06 (0.02-0.09)	0.26								
miR-4423-3p	0.18 (0.12-0.25)	0.32(0.20-0.44)	0.32 (0.24-0.40)	0.03								

Data are shown as mean expression (95%CI)

Table 10. Expression levels of the miRNA candidates based on Overall survival at T0, T1, and T6.

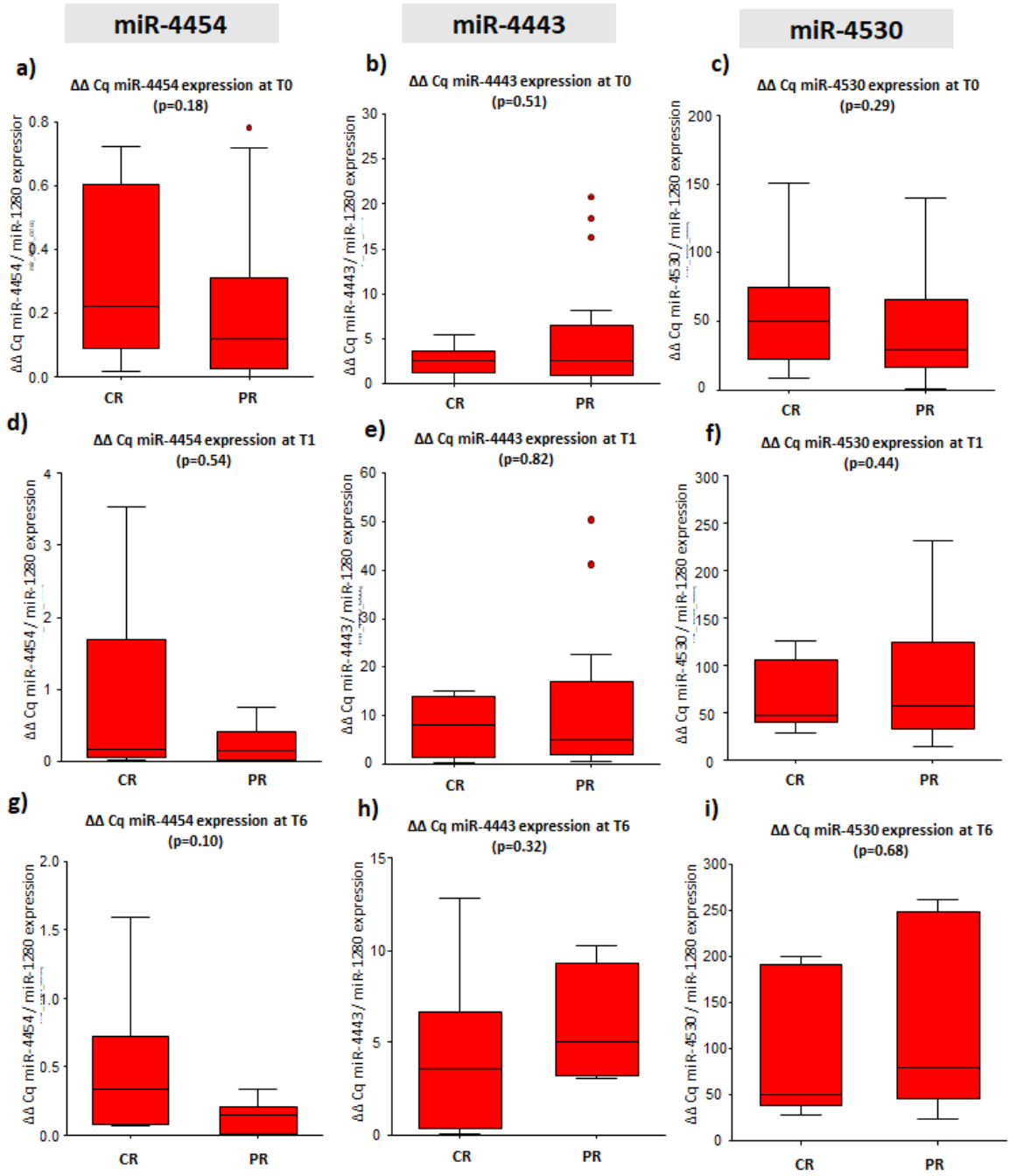


Figure 19. Mean ΔΔCq expression of miR-4454, miR-4443 and miR-4530 inCR and PR in TACE-treated patients.

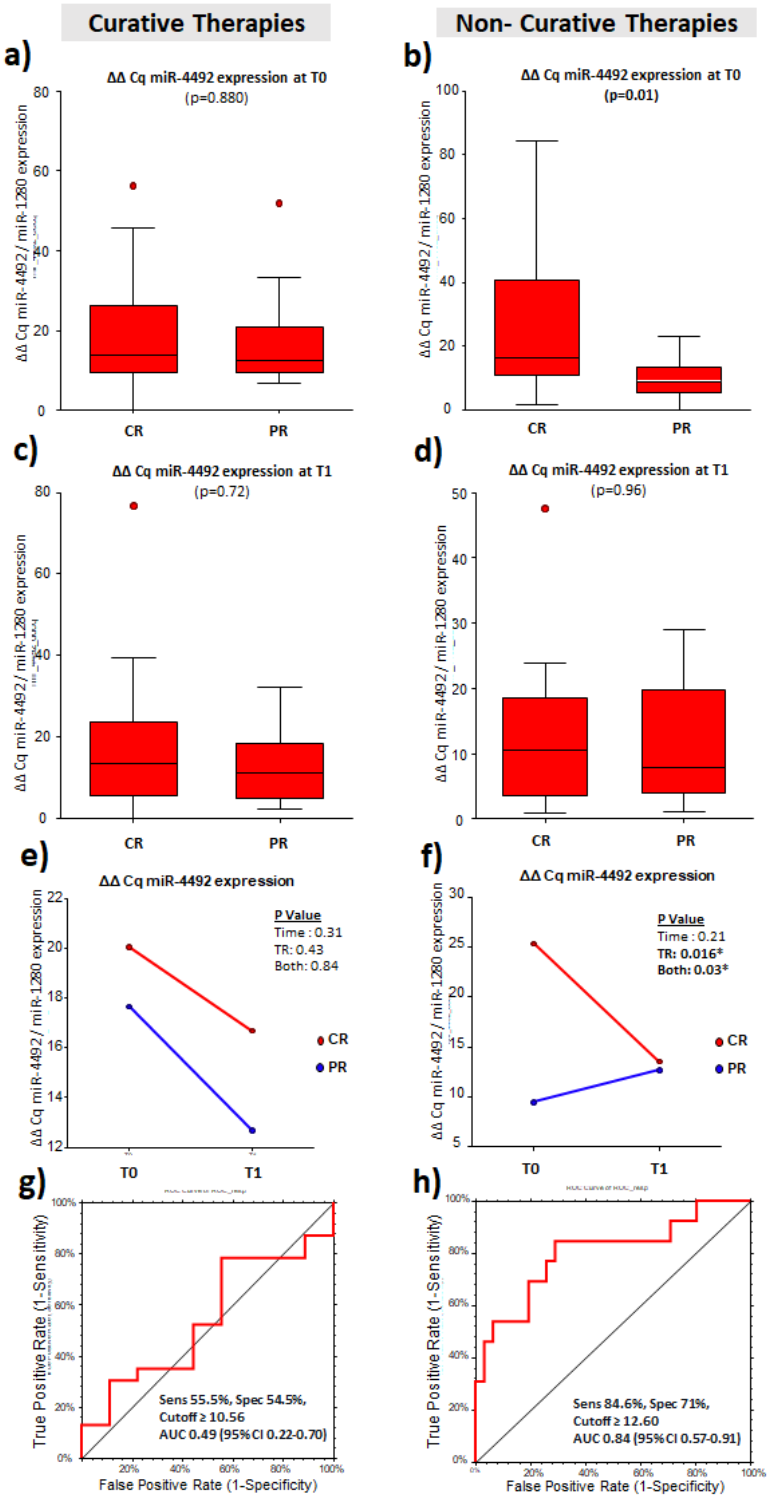


Figure 20. The differential expression of miR-4492 between CR and PR for curative therapies at T0 (a) and T1 (c) and TACE at T0 (b) and T1 (d). The pattern of expression of miR-4492 at each time points shown for curative therapies (e) and TACE (f). Receiver operating Curve (ROC) analysis of miR-4492 was performed for curative therapies (g) and TACE (h).

4.2.5. Serum miRNAs are significantly associated with overall survival before treatment

In the discovery phase, four miRNA (miR-335-3p, miR-3185, miR-4507, and miR-4423) were associated to OS before treatment. In the validation phase, patients were divided into three groups, according to the survival time (months): OS <12, OS 12-24 and OS >24. We observed a significantly different expression of miR-4507 (p=0.00037), miR-4423-3p (p=0.02), and miR-3185 (p=0.014), while miR-335-3p resulted not statistically significant (p=0.85) between three groups of patients at T0 **(Table 10).** The mean ΔΔCq expression of miR-4507 was extremely low in OS <12 months, 22.4 times higher in OS 12-24 months, and 25 times higher in OS >24 months **(Fig. 21a).** The expression of miR-4423-3p was 2 times higher in OS 12-24 months and OS > 24 months **(Fig. 21d).** Serum miR-3185 levels gradually increase according to the longer OS, being 1.4 fold times higher in OS 12-24 months compared to OS <12 months and 1.7 times higher in OS >24 months compared to OS <12 months **(Fig. 21g).** At T1 and T6 however, there is no miRNAs reaching a statistical significance when comparing the three OS groups **(Table 10).**

Since we are interested to discover the predictor of overall survival in HCC patients before initiation of therapies, our miRNA candidates were further analyzed using a Kaplan-Meier survival analysis. Patients were divided into Low *vs* High miR expression using the mean expression value of the group of patinets with OS<12. Higher expression of mir-4507 was significantly associated with longer OS with HR of 1.89 (95% CI 1.10-3.27, p=0.016) **(Fig. 21b).** When considering also the survival rate of patients who received no treatments (n=10), patients with higher expression of miR-4507 had 4.25 times chance of longer survival compared to the untreated ones (HR 4.25, 95% CI 1.22-14.83, p<0.0001) **(Fig. 21c).** Similarly, higher expression of miR-3185 was associated with 2.02 times longer OS at T0 (HR 2.02, 95% CI 1.10-3.73, p=0.0086) **(Fig. 21h).** Highest differences in the two groups of patients were mantained up to 4 years. Interestingly, patients with low miR-3185 expression had comparable survival time to untreated patients (p=0.06) **(Fig. 21i).** Kaplan-Meier estimates for miR-4423-3p were not statistically significant when comparing Low *vs* High miR-4423-3p groups **(Fig. 21e, 21f).**

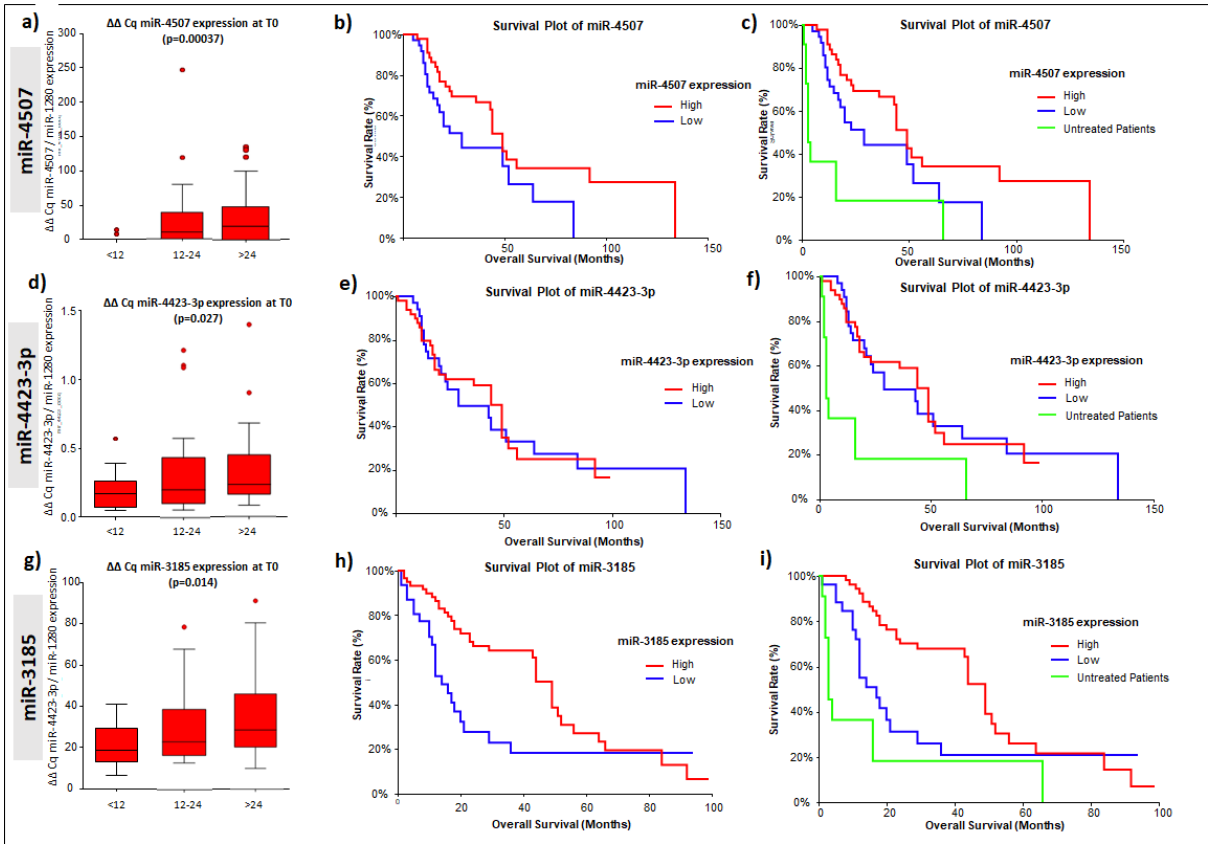


Figure 21. The differential expression of miR-4507 **(a),** miR-4423-3p **(d)**, and miR-3185 **(g)** between OS <12, OS 12-24 and OS >24 at T0, T1, and T6. Kaplan-meier survival analysis by log-rank test between high and low expression of miR-4454 and untreated patients of miR-miR-4507 *(p<0.001)*, **(b,c),** miR-4423-3p (*p=0.02)* **(e,f)**, and miR-3185 *(p=0.014)* **(h,i)** at T0 for patients receiving curative therapies.

4.2.6. Serum miRNAs are significantly associated with disease-free survival before treatment with curative therapies

Three serum miRNA candidates (miR-1246, miR-4454 and miR-4530) associated to DFS where further validated in further 105 serum samples. Patients were separated into two groups of DFS ≤ 6 months and DFS >6 months. The mean ΔΔCq expressions of all miRNAs candidate at T0, T1, and T6 are summarized at **Table 11.**

The expression of MiR-4454 (p=0.03) and miR-4530 (p=0.015) at T0 were significantly higher in patients with DFS>6 months specific for patients receiving curative therapies **(Fig. 22a, 22b).** Unfortunately, we were not able to confirm the role of miR-1246 as a predictor of

DFS in this phase. We calculated the AUC of ROC curve for both miRNAs at T0 to validate their discriminatory potential. Using a cut-off of Cq ≥ 0.20, miR-4454 distinguished patients with longer from patients with DFS< 6 months, with a sensitivity and specificity of 67% and 64%, respectively (AUC= 0.73 (95% CI 0.50-0.86), p=0.005) **(Fig. 22c).** Similarly, at a cut-off of Cq ≥ 53.9, miR-4530 reached a sensitivity and specificity of 70% and 80%, respectively (AUC = 0.78 (95% CI 0.56-0.90), p= 0.0003) **(Fig. 22d).** The combination of mir-4454 and miR-4530 had better performances to distinguish the two groups of patients. At a cut-off of > 0.53, the sensitivity increased to 79% while the specificity corresponded to 72%(AUC= 0.81 (95% CI 0.59-0.82) p=0.008) (Logit model formula: -1.63 + 2.95 * miR-4454 + 0.00 * miR-4530) (**Fig. 22e).** Despite the improvement given by the combination of the two miRNAs, miR-4530 alone might be a potential as single miRNA to predict DFS in patients candidate for curative therapies.

Table 11. Expression levels of the miRNA candidates based on disease-free survival at T0, T1, and T6.

	T0			T1			T6		
miRNA	**DFS ≤ 6 months**	**DFS > 6 months**	**P value**	**DFS ≤ 6 months**	**DFS > 6 months**	**P value**	**DFS ≤ 6 months**	**DFS > 6 months**	**P value**
miR-1246	5.83 (1.14-3.52)	5.07 (3.44-6.70)	0.54	20.68 (11.49-29.86)	19.79 (10.91-28.67)	0.92	5.08 (1.76-8.41)	6.86 (4.05-9.67)	0.17
miR-3185	30.83 (24.49-37.18)	30.46 (23.71-37.21)	0.71						
miR-4492	14.73 (10.43-19.04)	19.05 (13.34-24.73)	0.052	12.36 (8.92-15.80)	16.95 (9.87-24.04)	0.51			
miR-4454	0.19 (0.13-0.25)	0.31 (0.20-0.43)	**0.05***	0.19 (0.12-0.25)	0.53 (0.25-0.81)	0.057	0.24 (0.01-0.46)	0.69 (0.22-1.16)	**0.046***
miR-4530	62.43 (34.67-90.17)	72.45 (50.95-93.95)	**0.02***	85.91(60.62-111.12)	71.39 (45.59-97.18)	0.23	106.03 (61.36-150.71)	78.68 (23.07-134.29)	0.16
miR-4443	4.93 (3.21-6.66)	2.62 (1.72-3.52)	0.06	7.34 (3.72-10.95)	7.16 (4.35-9.98)	0.64	4.32 (2.74-5.91)	7.91 (-1.9-17.75)	0.81
miR-4507	24.65 (12.25-37.06)	28.69 (15.42-41.97)	0.68						
miR-335-3p	0.10 (0.03-0.17)	0.08 (0.02-0.13)	0.51						
miR-4423-3p	0.31 (0.23-0.38)	0.29 (0.20-0.40)	0.56						

Data are shown as mean expression (95%CI)

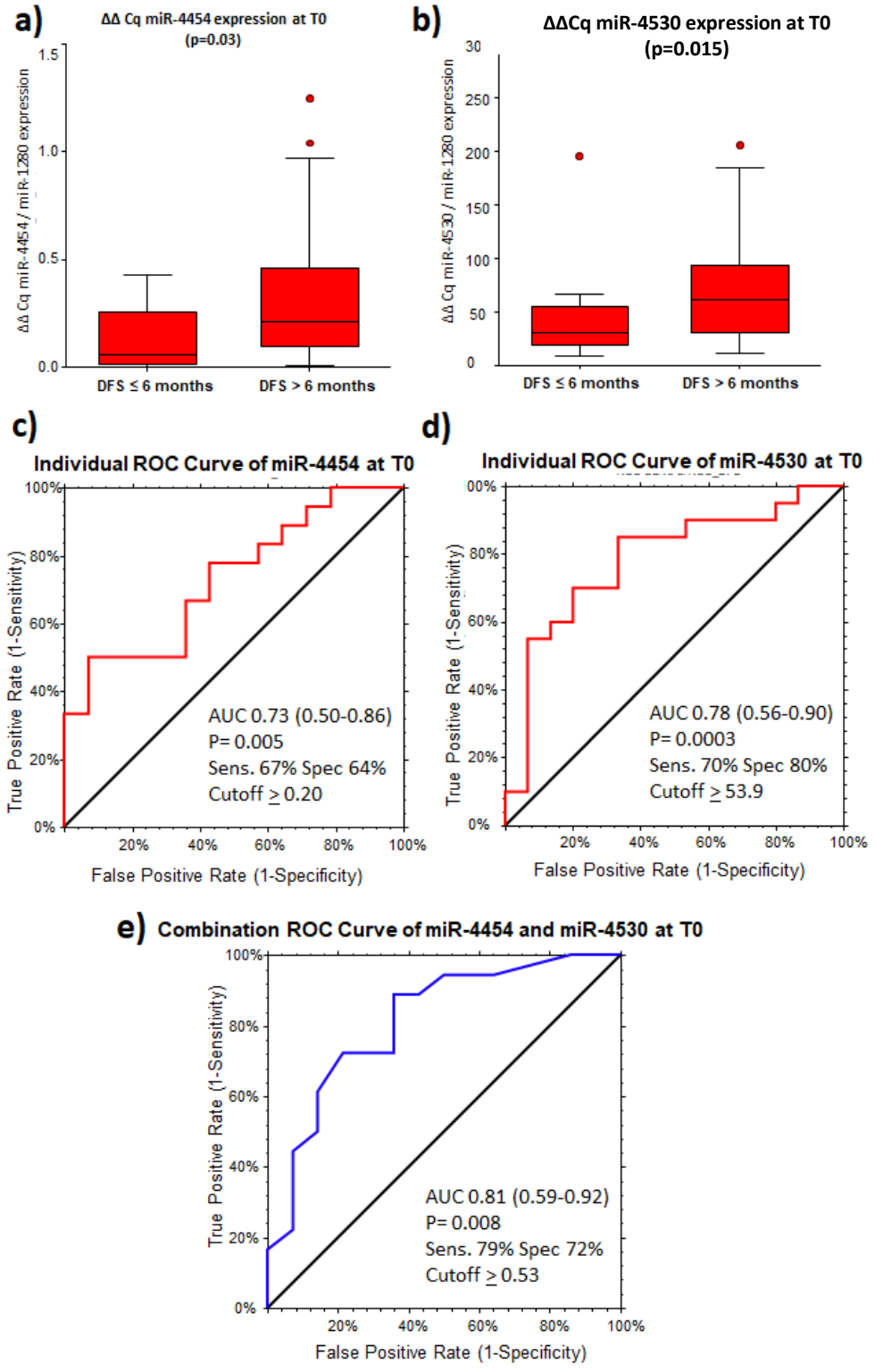


Figure 22. Mean ΔΔ Cq expression of miR-4454 **(a)** and miR-4530 **(b)** in patients with DFS ≤ 6 months and DFS >6 months at T0. Receiver operating Curve (ROC) analysis of miR-4454 (**c),** miR-4530 **(d)** and in combination **(e).**

4.3. TASK 3 - Validation of miRNA Targets and Cellular Pathway

From the previous two tasks, we identified several miRNAs as possible predictive marker or predictor of prognosis. However, there are no or lack of evidences regarding the presence of these miRNAs in liver tissue and their molecular role in HCC, including their putative targets and the involved pathway. Therefore, we analyzed the expression of our miRNAs candidate in HCC tumoral and distal tissue and conducted *in silico* and *in vitro* analysis to unravel the complete cellular pathways of our candidate.

4.3.1. MiRNAs differently expressed between tumoral and distal tissues in HCC

The expression of four miRNAs (miR-3197, miR-4492, miR-4454, and miR-3185) that were found to be significant predictors were further analyzed in ten paired tumoral and distal tissues from HCC patients **(Fig. 23).** The experiments were conducted for four selected miRNAs. MiR-3197 as predictive tool , miR-4492 in relation to TR in non-curative treatments, miR-4454 in relation to TR in curative treatments and DFS, and miR-3185 in relation to OS. From qRT-PCR analysis, all miRNAs were expressed in either tumoral (T) and distal (D) liver tissues. Based on the Cq expression, We noticed low expression of all miR-3197, miR-4492, and miR-4454 in tumoral compared to distal tissue in all ten patients. While not clear differences where found for miR-3185.

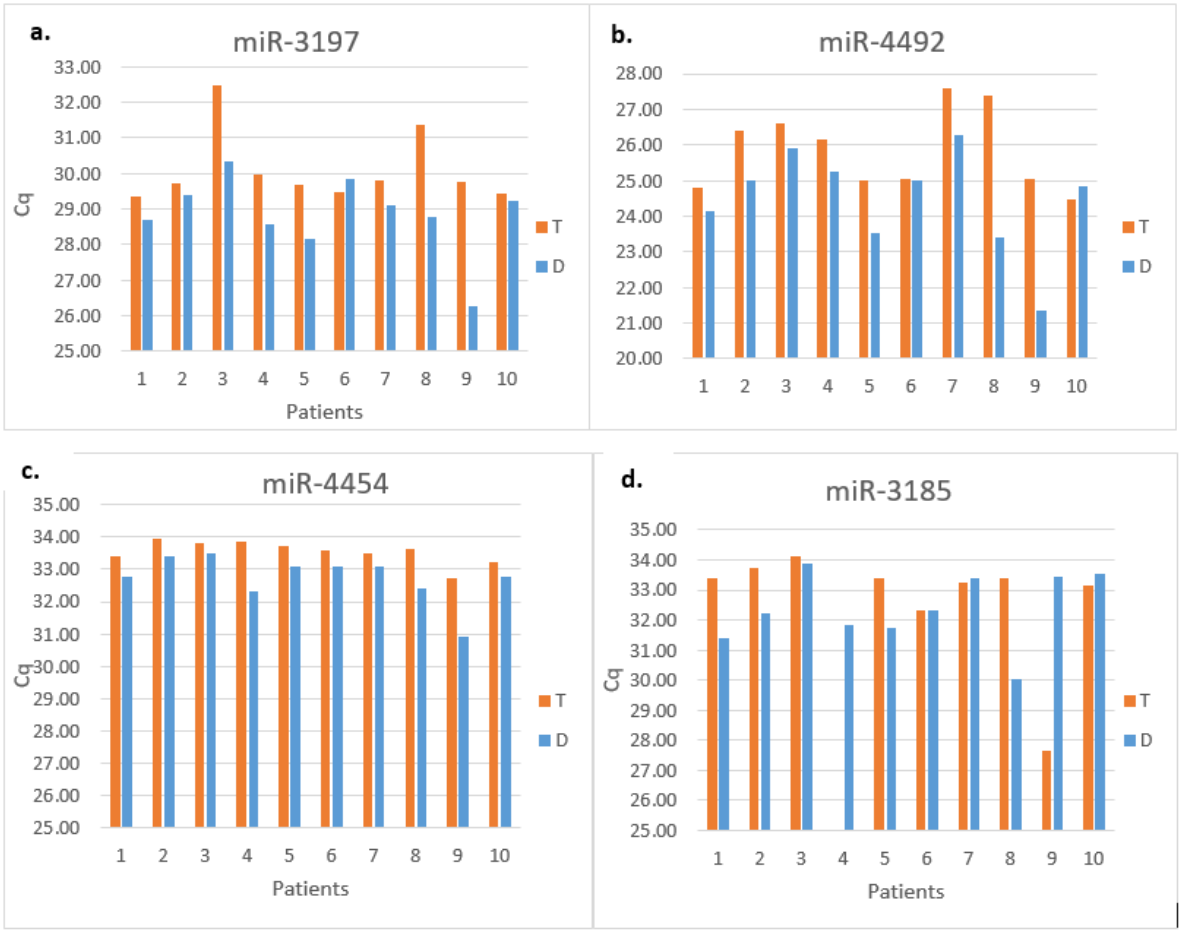


Figure 23. Cq expression of miR-3197 **(a),** miR-4492 **(b),** miR-4454 **(c)** and miR-3185 **(d)** in tumoral (T) and distal (D) tissues from HCC patients.

4.3.2. *In Silico* Prediction of miRNAs gene target

Since there are general lack of information regarding these miRNAs, we decided to investigate their possible role in cancer cells by identifying their target genes and study their involvement in HCC in an in vitro model. Three miR-target prediction tools (TargetScanHuman, miRDB and DIANA TOOLS) were used in order to obtain a list of possible miRNA targets for each miR candidate. A total of 118 genes were discovered to be predicted targets from at least two out of the three online tools used. 24 genes were predicted as targets for miR-4492, 35 genes for miR-3197, 45 genes for miR-3185 and 14 genes for miR-4454. All the shared genes for each miRNAs were then ordered based on the mean of their cumulative rank for each databases. In order to select miRNA target genes as candidates for *in vitro* experiments. We further proceed to schematic literature search **(Fig. 24)** using Pubmed and GeneCards to

collect general information about each genes. 52 genes were discarded in this step because they have no documented or suspected role in any cancer-related pathway. The remaining 60 genes underwent a selection process to met one of the target selection criteria consisting of: 1) evidences of their involvement in any cancer pathways, 2) Novelty; target genes never studied in HCC settings, 3) Possible release in plasma or serum. 16 genes were then selected as our final set of predicted target genes for validation study on *in vitro* system **(Table 10).**

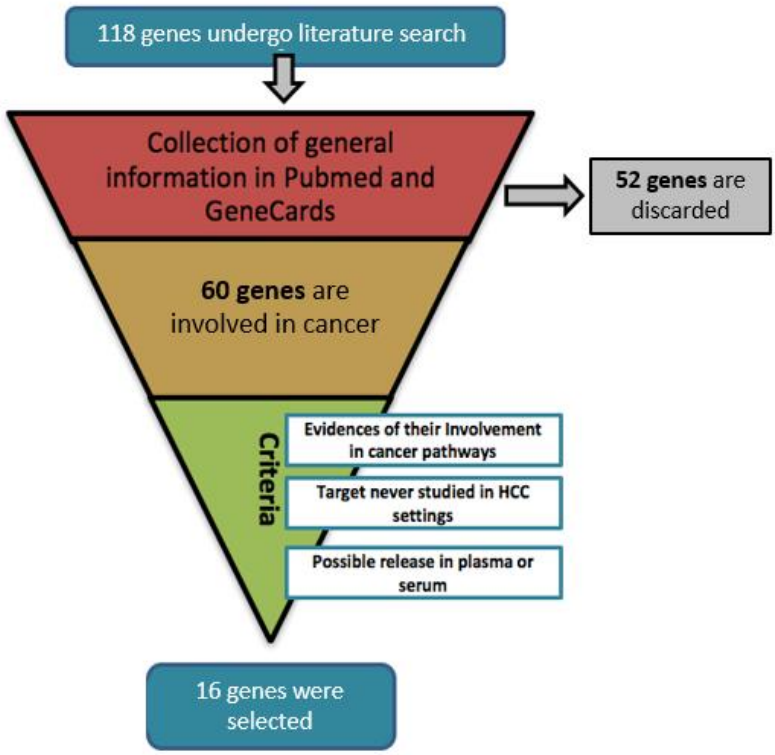


Figure 24. The schematic literature search for the predicted target genes.

miR-3197	**miR-4492**	**miR-4454**	**miR-3185**
NUPR1	KLK4	BAG5	ERCC1
IGFBP5	JMJD8	EIF4A2	
CDKN2D		DLG5	

FOXP2
MXD1
HOXC6
CTNNBIP1
NLRP3
NDEL1

Table 10. Predicted target genes for each miRNAs for validation study in cellular model.

4.3.3. Candidate genes expression in tumoral and distal tissues.

We proceed further to analyze the expression all three target genes on the same ten paired tumoral and distal tissues from HCC patients **(Fig. 25).** Among the prospective target genes for each miRNA, the three candidates of miR-4454 shown an opposite trend of expression in comparison to the down-regulation of miR-4454 in tumoral tissues. In particular, DLG5 was up-regulated in tumoral tissues in 90% of patients, EIF4A2 in 80% and BAG5 in 60% of patients. For miR-3197, NLRP3 and CTNNBIP1 were upregulated in 80 and 60% of tumoral tissues compared to distal part, showing some correlation between miRNA and the target genes. However, none of the target genes of miR-3185, and miR-4492 shown any significant trend of expression among the tumoral and distal tissue samples. Therefore, for this project, we chose miR-4454 for the following *in vitro* target validation phase, since its expression was both concordant in all studied tumoral samples and generally opposite to its gene candidates.

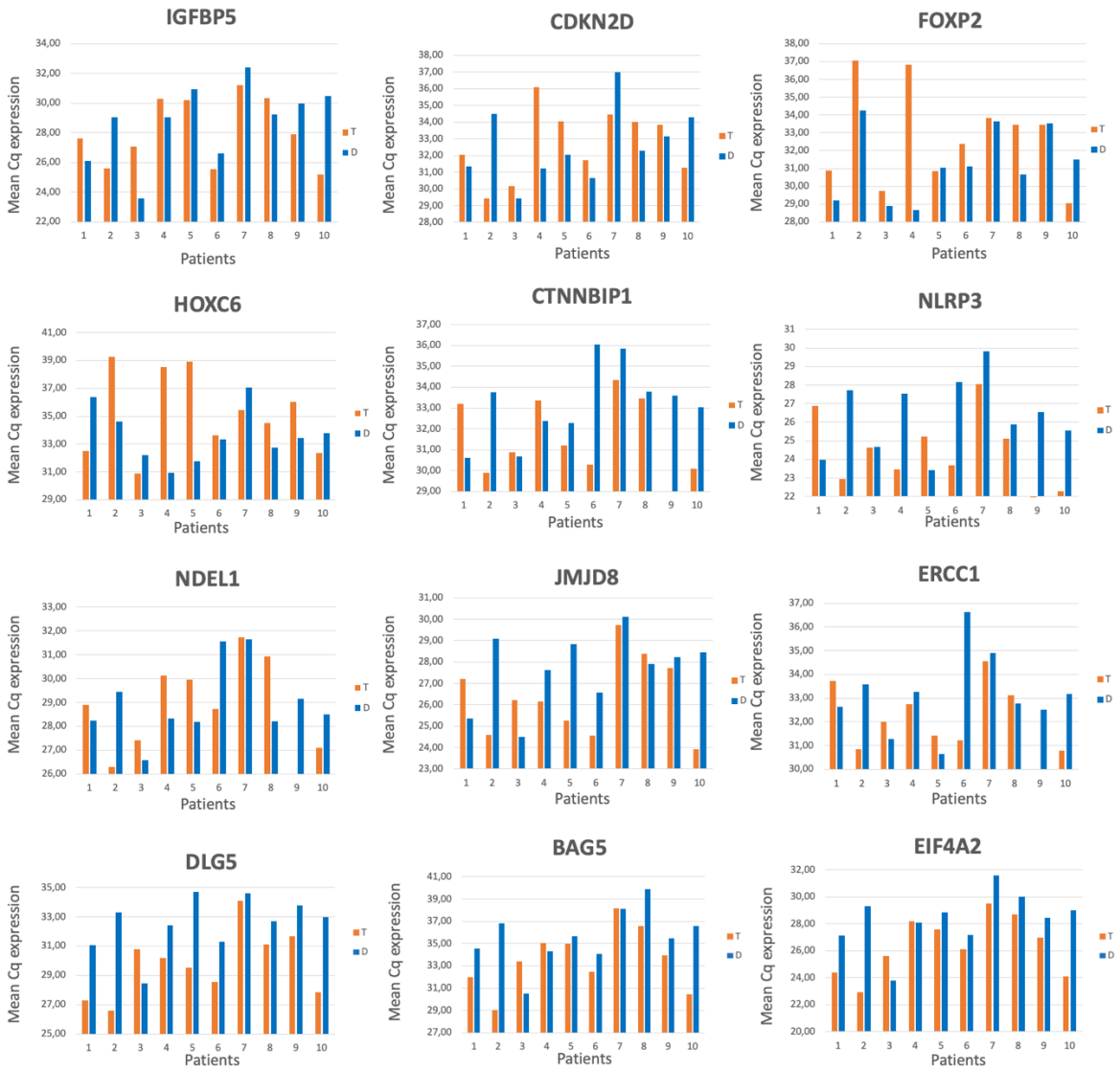


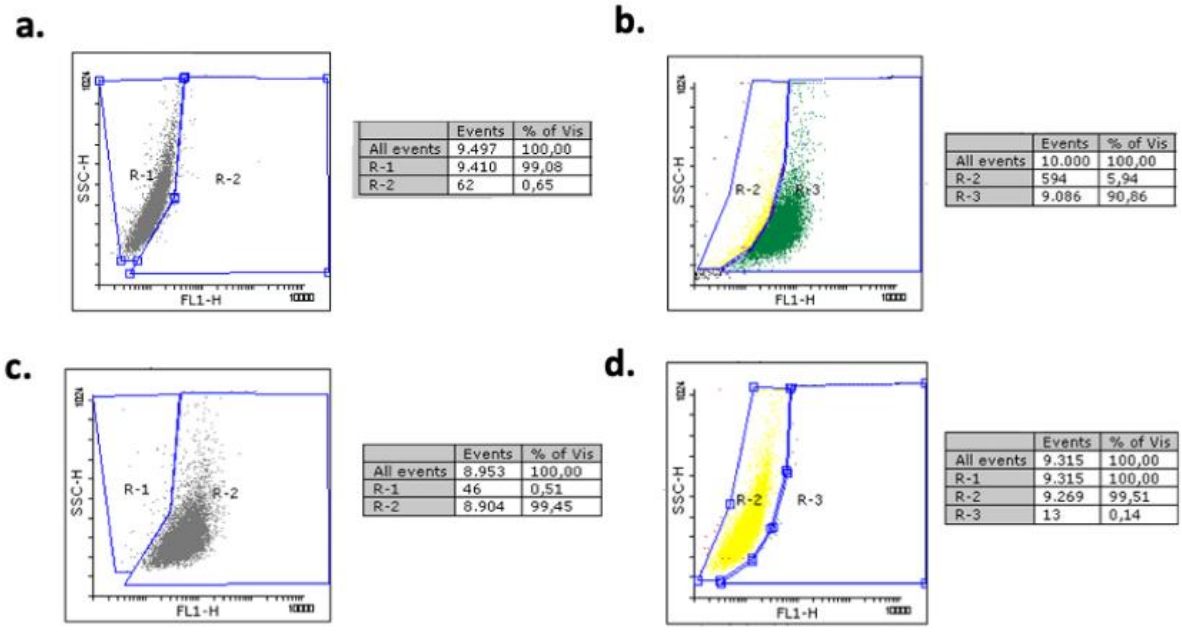
Figure 25. Mean Cq expression of target gene candidates of miR-3197, miR-4492, miR-3185, and miR-4454 in tumoral (T) and distal (D) tissues from HCC patients.

4.3.4. *In vitro* miRNA target validation study

In order to validate the predicted target genes of our miRNAs of interests, transient transfection experiments were performed in a HCC cell line. In this part of the project, miR-4454 and its predicted target genes were analysed in JHH6 cells. Following transfection with miRNA mimic, we aimed to investigate the modulation of of target genes in HCC-derived cells, verifying miR-4454 role as post-transcriptional regulator.

4.3.4.1. Transfection efficiency

The efficiency of the transfection complex delivery within cells was evaluated by transfecting JHH6 (6.000 cells/cm^2 and 8.000 cells/cm^2) with 50nM of FiTCconjugated miR. After 24 hours, FACS analysis was performed to examine the fluorescence signal emitted by the internalized FITC molecule that represents the rate of miRNA mimic transfected inside of the cells. As shown in **Fig.26**, the percentage of fluorescent cells at 6.000 cells/cm^2 and 8.000 cells/cm^2 concentrations were 99.45% and 90.86%, respectively. Therefore, both cell concentrations were considered suitable for the following *in vitro* experiments.



	Events	% of Vis
All events	9.497	100,00
R-1	9.410	99,08
R-2	62	0,65

	Events	% of Vis
All events	10.000	100,00
R-2	594	5,94
R-3	9.086	90,86

	Events	% of Vis
All events	8.953	100,00
R-1	46	0,51
R-2	8.904	99,45

	Events	% of Vis
All events	9.315	100,00
R-1	9.315	100,00
R-2	9.269	99,51
R-3	13	0,14

Figure 26 – FACS analysis of cell fluorescence after miR-4443 mimic – FITC transfection. Control (untreated) at 6.000cells/cm concentration **(a).** Transfected sample at 6.000 cells/cm concentration **(b).** Control (untreated) at 8.000cells/cm concentration **(c).** Transfected sample at 8.000 cells/cm concentration **(d).**

4.3.4.2. MiR-4454 over-expression downregulate DLG5 *in vitro*

Three replicates of transient transfection of miR-4454 or scramble miRNA (negative control) were performed in JHH6 cells for 48 hours. As shown in **Figure 27a**, following the transfection, we detected a 7 Cq and 9 Cq differences in miR-4454 expression in cells treated with 25nM and 50nM of miR-4454 mimic, respectively, compared to both untreated JHH6

cells and the negative control. There is no difference between both untreated cells and negative control. (p>0.05).

To confirm DLG5, EIF4A2, and BAG5 as miR-4454 target genes, we analysed their expression in miR-4454 mimic-transfected cells. After 48 hours of transfection, we observed 25% of decrease of the DLG5 expression (p=0.05), in cells treated with 25nM of miR-4454 mimic, and 36% of decrease (p=0.004) in cells treated with 50nM of miR-4454 mimic **(Fig.27b)**, thus indicating that miR-4454 targets DLG5 at RNA level. Meanwhile, there were no significant differences observed in the expression of EIAF4A2 **(Fig. 27c)** and BAG5 **(Fig. 27d)** transcripts in cells treated with miR-4454 mimics compared to control.

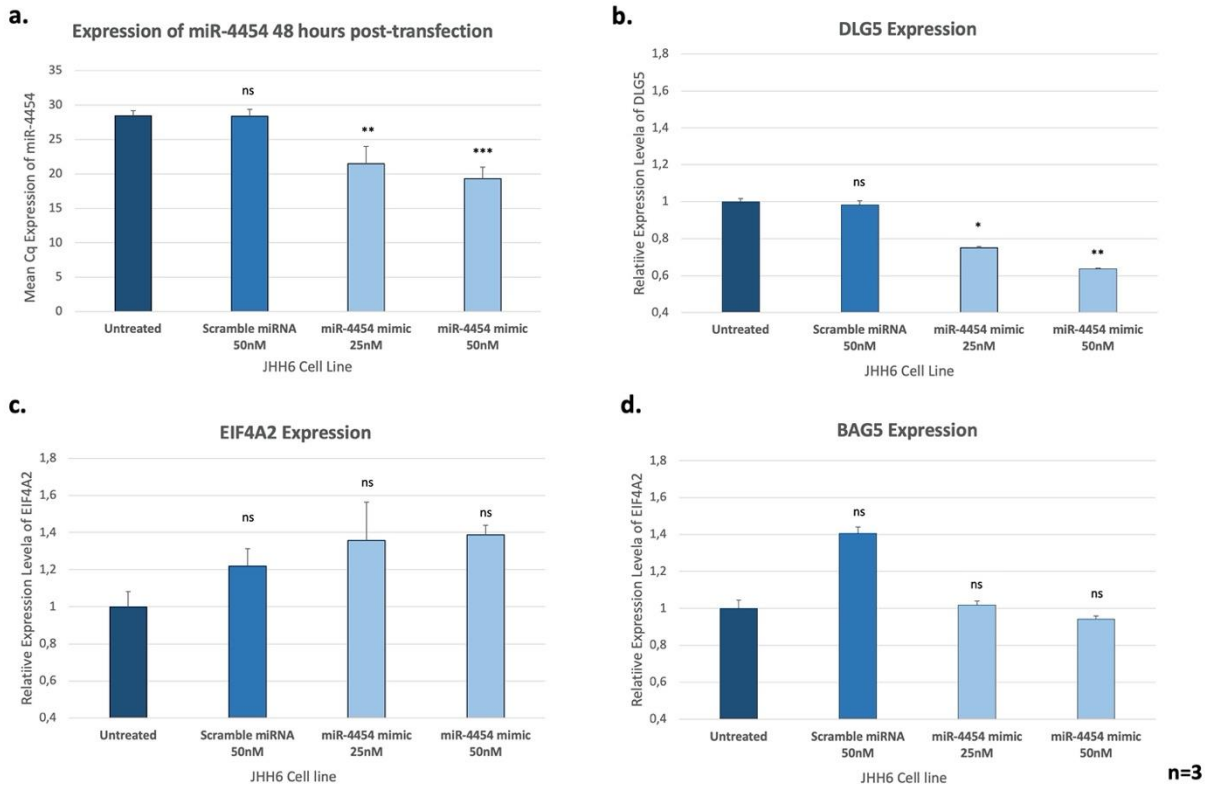


Figure 27 – MiR-4454, BAG5, DLG5 and EIF4A2 expression detected after miRNA-mimic transient transfection at 48 hours. Mean Cq expression of miR-4454 from untreated JHH6 cell line, and JHH6 cell line transfected with 50nm scramble miRNA (negative control), 25nM and 50nM of miR-4454 mimic **(a)**. Relative expression of DLG5 **(b),** EIF4A2 **(c),** and BAG5 **(d)** in untreated JHH6 cell line, and JHH6 cell line transfected with 50nm scramble miRNA (negative control). Each experiments were repeated three times and similar results were obtained. Ns= not significant; *p<0.05; **p<0.01; ***p<0.001. The data are presented as mean ± SD.

4.3.4.3. MiR-4454 represses the HCC cell migration

Since the putative role of DLG5 in cell migration we decided to perform a a scratch-wound assay to further investigated the role of miR-4454 in the migration of HCC cells. JHH6 cells in 24-wells plate were transfected with miR-4454 mimic or scramble miRNA (negative control) and the scratch was performed 24 hours after transfection. We observed that miR-4454 over-expression was significantly inhibited the migratory capacity of the cells after 24 hours of scratch injury (48 hours post transfection) compared to the untreated cells and negative control **(Fig.29a).** In fact, from the two independent experiment performed, cells that were transfected with 25nM of miR-4454 mimics had 25% less wound closure compared to untreated and negative control, while transfection with 50nM of miR-4454 mimics decelerate the wound closure and migratory potential of JHH6 cells to 50% compared to control **(Fig. 29b).** Cells transfected with miR-4454 mimics were all returned to 100% wound closure in 72 hours post-transfection.

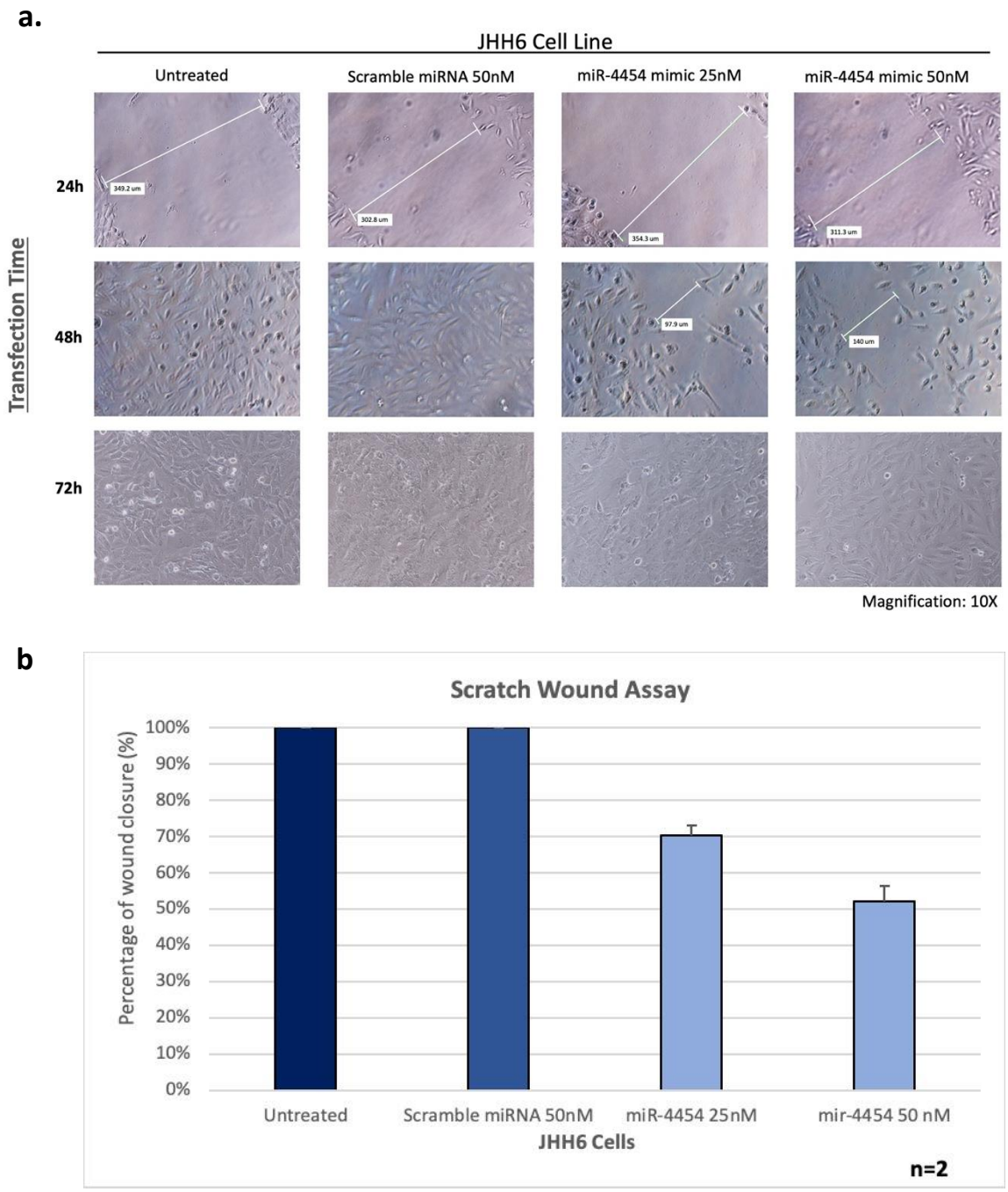


Figure 29 – MiR-4454 inhibits JHH6 cells migration. Wound-scratchs assay were performed to assess the migration rate of JHH6 cells transfected with 25nM and 50nM of miR-4454 mimics **(a)**. Photographs were taken at the indicated time points after scratch injury. The percentage wound closure were quantified by measuring the distance between marginsin the injured region **(b).** Experiments were performed in two replicates. The data are presented as mean ± SD of two independent experiments

4.3.5. DLG5 expression is upregulated in HCC tissues *in silico*

The expression of DLG5 was explored in StarBase database (http://starbase.sysu.edu.cn/) showing a clear DLG5 increased expression in tumoral liver tissues, in comparison to healthy liver (**Fig. 30**). In addition, data from the Human Protein Atlas (https://www.proteinatlas.org/ENSG00000151208-DLG5/pathology) showed the relation between a higher DLG5 expression and a lower survival probability (**Fig. 31)**, assuming its oncogenic role within cancer.

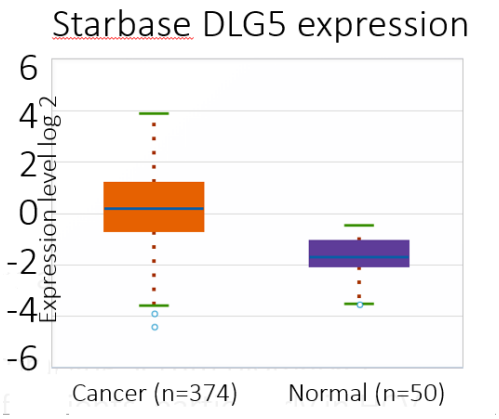


Figure 30 - DLG5 expression in two cohorts of tumoral and helatly liver tissues (Taken from Starbase database, http://starbase.sysu.edu.cn/) 2020)

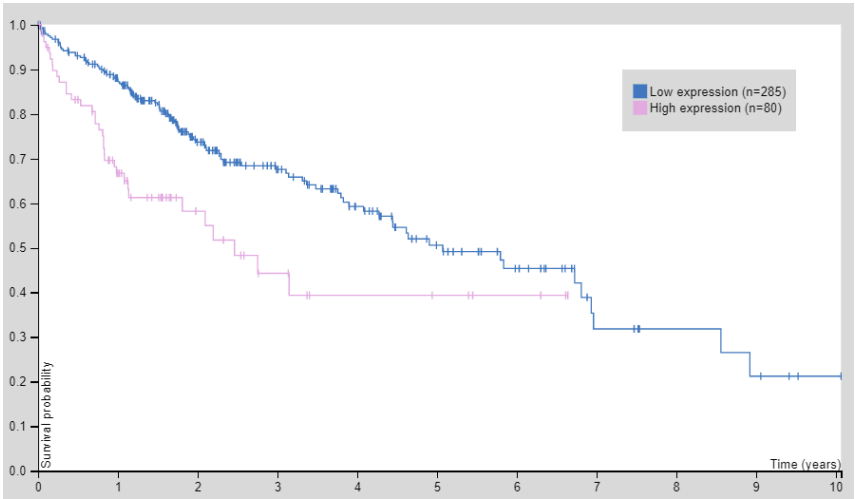


Figure 31 – Survival probability related to low and high DLG5 expression in HCC tissues (Taken from The Human Protein Atlas: https://www.proteinatlas.org/ENSG00000151208-DLG5/pathology)

Chapter 5

Discussion

Hepatocellular carcinoma represents the fourth leading cause of cancer-related death worldwide and remains as one of the most challenging types of cancers due to the late diagnosis and poor prognosis after treatments. HCC grows silently with non-specific set of signs and symptoms, thus making diagnosis in early stages remain difficult. Unlike most solid cancer, imaging techniques have significant drawback in early stages of HCC, and the current non-invasive biomarkers have low sensitivity and specificity. From the molecular standpoint, multiple and diverse etiological risk factors mirror the pattern of molecular diversity encountered in the disease. This heterogeneity derives from different key mutations during the complex process of carcinogenesis. The different molecular profile, characterizing every single patient, might further contribute to the phenotypic and clinical manifestations of the tumor itself while also play a crucial role on the inefficacy of current treatments. Moreover, despite the current advancement in HCC management, the fact that most patients are diagnosed at advance stages made curative treatments are not feasible for the great majority of HCC cases. Non-curative therapies and molecular-targeted therapies for advance stages have poor long-term outcome, due to multi-drug resistance or to the heterogeneous molecular profile of tumor previously described.

To overcome these issues, we aimed to identify novel non-invasive biomarker, such as circulating miRNAs, to use as predictive tools for early HCC detection and for prognostic predictors for a better patient's management. In addition, we aimed to explore the molecular pathways regulated by the miRNAs working as HCC-related biomarkers. These aims were addressed in three different tasks consisting of:

- TASK1: Serum miRNA biomarkers for early hepatocellular carcinoma occurrence following direct-acting antivirals treatment.
- TASK2: Serum miRNAs as Prognostic Biomarker after HCC Treatment
- TASK 3: *In vitro* validation of miRNA targets and cellular pathway, to clarify the role of miRNAs candidates.

5.1. Serum miRNA biomarkers for early hepatocellular carcinoma occurrence following direct-acting antivirals treatment

We conducted the first part of this project to test the potential of serum miRNA as predictive biomarker from a specific cohort of HCC occurrence after DAA treatment in cirrhotic patients with chronic hepatitis C. Despite the improvement in the Hepatitis C treatments, some recent studies raised concerns about the persistence of HCC risk after DAA therapy in patients with cirrhosis [243–246]. In addition, tumors occurred after DAAs treatments were described as more aggressive, with multifocal and metastatic involvement [246,247]. Other studies still weighing that the patients with SVR remains to have lower risk compared without SVR, even though there is a 4.2% *de novo* risk of HCC in HCV-related decompensated cirrhosis treated by DAA. The only prospective study was from *ANRS CO12 CirVir cohort* enrolling 1270 CHC patients over six years in France, that shown no statistically significance increase in the risk of HCC development after DAA treatment [248].

Whether or not DAA is contributing to the development of tumors still remain as major controversy [27]. In fact, there is no clear evidence of any proven contribution of DAAs in the development of tumor in predisposed patients. Among the pathophysiological hypothesis, the most appealing one based on the literature review conducted by Villani *et al.* (2018) suggest the involvement of immune cell alteration, imbalance of cytokine network, and angiogenesis that could explain this phenomenon [29]. The reduction of immune surveillance as a possible enhancer of tumor development might play a role, as it was described that several immune mediators have found to be differently regulated in both patients that developed HCC or not after DAA treatment [29]. Other hypothesis formulated by Faillaci *et al.* (2018) suggested that the DAA-mediated increase of VEGF might contribute to tumor growth in cirrhotic subjects with already over-expressed Angioprotein-2 in liver tissues [249]. To contribute in this debate, we performed a circulating miRNA profile of cirrhotic patients treated with DAA before and after therapy initiation, in order to identify miRNA biomarkers predicting the risk of HCC occurrence. Our group of patients consisted of patients developing HCC after DAA treatment (HCC+) compared to patients not developing HCC (HCC-) within 12 month after SVR.

In the discovery phase, through miRNA array profiling analysis we identified 9 miRNAs, consisting of miR-1207-5p, miR-1275, miR-3197, miR-4443, miR-3178, miR-483-5p, miR-4706,

miR-4793-3p and miR-1246, as significantly and differently expressed between the groups HCC+ and HCC− before DAA treatment (T0), thus suggesting the possibility of pre-existing commitment through the development of HCC before DAA initiation. The differences in the miRNA expression were also observed at T1, however involving different miRNAs consist of miR-1180-3p, miR-1228, miR-4329 and miR-4484. To confirm these observations we further investigated the expression by RT-qPCR of the 13 miRNA candidates in a cohort of 60 matched samples, (30 HCC+ *vs*. 30 HCC−) obtained at T0 and T1. The time difference of the mean obtained from repeated measures of the selected miRNAs in HCC+ *vs.* HCC− patients, was confirmed by bootstrap cross-validation analysis on 1000 samples, evidenced the significant changes in miR-3197.

We also reported a similar level of miR-3197 expression between HCC-and healthy individuals, but a significant trend of downregulation when comparing HCC+with the other two populations (HCC− and healthy individuals). Therefore, we hypothesized that miR-3197 might play a role as a tumor suppressor miRNA for HCC development and the upregulation of miR-3197 in cirrhotic patients might represent a pre-existent marker for specific HCC risk. Indeed, as confirmed using the ROC curve analysis, miR-3197 performancies were not satisfactory when discriminating HCC− and healthy individuals, but it has a discriminatory potential between HCC+ cirrhosis and the other two groups. The highest values of AUC, sensitivity, and specificity (AUC 0.78, 79.0%, and 70.0%, respectively) were obtained when comparing HCC+and HCC−, showing the potential of miR-3197 as not only a diagnostic biomarker, but also a risk predictive biomarker that can be utilized for surveillance biomarker in cirrhotic patients.

Interestingly, we found consistent results when testing the miR-3197 expression in paired HCC tissues. MiR-3197 was found to be always down-regulated in tumoral tissue compared to distal tissue from in ten human samples that we were analyzed, implying a possible liver origin of miR-3197. Parallel with this result, one available study for miR-3197 coming from Sayagues *et al.* (2016) described the presence of miR-3197 in liver metastatic tumor tissue from colorectal cancer [250]. Based on these evidences, miR-3197 could represent as a promising serum biomarker candidate for the identification of patients at risk after DAA treatment, particularly in HCV-related liver cirrhotic patients. This imply the potential of miRNA as a predictive biomarker especially in a high-risk setting such as DAA-

treated patients. The relevance of miRNA as biomarkers in this particular clinical setting was evidenced in another recently published study by Itami-Matsumoto *et al* (2019) conducted in a cohort of SVR-HCV patients that develops HCC recurrence, in which they identified several exosomal miRNAs related to HCC onset after HCV eradication, thus suggesting the potential role of miRNAs as a predictive biomarker in this high-risk population [251]. Neverthless, considering that the etiology of all of our cirrhotic patients is chronic hepatitis C virus (HCV), it would be interesting to further analyze the expression of miR-3197 in other etiologies to assess whether this microRNA is specifically dysregulated in HCV-derived HCC.

To our knowledge, this is one of the first longitudinal studies assessing the role of miR-3197 in HCV patients, before and during DAAs therapy, as potential biomarker for the early HCC occurrence. Several limitations are present in this study consisting in the small sample size and the peculiarity of the group of the patient analyzed. However, the novelty of this miRNA open a question regarding its role in HCC molecular pathway. Through an *in silico* target prediction analysis we discovered 9 genes as predicted targets of miR-3197 to be involved in some cancer-related pathways: *NUPR1, IGFBP5, CDKN2D, FOXP2, MXD1, HOXC6, CTNNBIP1, NLRP3,* and *NDEL1.* NLRP3 for example, is a component of the inflammasome that had been described to be involved in tumor pathogenesis and had been strongly related to cancer survival, invasiveness, and resistance to treatment in several *in vivo* model from various set of cancers, including HCC [252–254]. In lung cancer, *NLRP3* had been shown to initiate chronic inflammation leading to inflammatory response and eventually cancer progression [252]. In breast cancer, NLRP3 activation in tumour-associated macrophages facilitates a favorable microenvironment for mammary carcinoma development [252]. While in HCC, *NLRP3* is reported to promote apoptosis and inhibit proliferation in HCC cell lines, opening a question whether mir-3197 might targeting *NLRP3* and involved in this pathway [254]. Another example is *IGFBP5*, that is positively associated with migration and invasion of tumor cells in colorectal cancer cancer [255]. However, no documented role about this gene in HCC, thus opening new perspectives regarding its involvement in liver cancer. Thus, validating these predicted targets of miR-3197 might be another key step to reveal the role of this miR in HCC, and more experimental data are needed to validate the interplay between those putative target genes and miR-3197.

5.2. Serum miRNAs as prognostic biomarker in HCC

Previous studies have identified multiple circulating miRNAs as prognostic biomarker in HCC. However, there are no longitudinal studies have considered and compared a panel of miRNAs as prognostic biomarkers in both curative and non-curative treatments. In the present study, we addressed the potential of serum miRNAs as prognostic biomarker after HCC treatment before therapy (T0), one month (T1), and six months (T6) after therapy in a cohort of 105 HCC patients receiving either curative (resection and thermoablation) or non-curative treatments (TACE).

Based on the previous results from the discovery phase using a microarray profiling analysis, 9 miRNAs, consisting of miR-1246, miR-3185, miR-4492, miR-4454, miR-4530, miR-4443-5p, miR-4423-3p, miR-4507, and miR-335-3p were significantly associated to different prognostic variables. Mir-4454, miR-4492, miR-4530 and miR-4443 were significantly associated with therapy response (TR). MiR-3185, miR-335-3p, miR-4423-3p and miR-4507 were associated with overall survival (OS). Mir-1246, miR-4454 and miR-4530 were associated with disease-free survival (DFS). To our knowledge, besides miR-1246, this is the first study describing the role of these miRNAs in HCC setting. To validate these observations we extended the analysis to a larger group of 105 patients by using qRT-PCR.

Considering the whole group of patients, miR-4454, miR-4492, and miR-4530 were significantly associated to therapy response, being higher in complete responders at T0. MiR-4454 was the only miRNA differently expressed at T6. Separately, in the patients receiving curative treatments, high levels of miR-4454 and miR-4530, and low level of miR-4443 were associated with better response to therapy at T0. ROC curve analysis confirmed the discriminatory potential of the three miRNAs in distinguishing CR from PR patients. However better performances were obtained by the combination of the three miRNA at T0, suggesting their potential use as non-invasive predictor of response for curative treatment before therapy initiation.

Interestingly, none of our miRNA candidates are found to be significantly different between CR and TR at T1. This might be due to the dynamic fluctuation of the miRNA levels after therapy, related to physiological status and/or to the specific treatment response [256]. Summerer *et al.* (2013) hypothesized that the observed significant changes of circulating miRNAs after therapy are likely to originate from the damaged tumor cells following

treatments [257] . Despite the absence of statistical significance at T1, the expression of miR-4454 and miR-4530 is higher in CR at all considered times. The observed differences might reflect the different molecular profile of the tumor. Distinct HCC molecular subtypes could express different miRNA levels, which might influence several critical steps in cancer pathways, such as invasion, apoptosis and other mechanism closely related to resistance to treatment. Thus, the low expression of miR-4454 and miR-4530 in PR patients might be related to some molecular mechanisms that significantly contribute to the response towards treatments.

Interestingly, the high expression of both miR-4454 and miR-4530 at T0 were also associated with better DFS, that is considered as a parameter to determine the magnitude of effectivity of the treatment. Thus, the different expression profile of miR-4454 and mir-4530 in both groups of patients (CR and longer DFS *vs.* PR and shorter DFS) before the treatment, might suggest their potential role as independent biomarker to predict the effectivity of treatment helping clinicians to define a better therapy strategy for the patient. Interestingly, patients having high levels of circulating miR-4454 have also longer OS, strengthening the importance of this miRNAs in several aspects of the disease.

Based on the current literature, none of miR-4454 and miR-4530 had been studied in relation to HCC. However in the contrary to our result, the high expression of circulating miR-4454 have been documented in two studies conducted in bladder cancer and melanoma [258,259]. Interestingly, only one study associated the high expression of miR-4530 with chemosensitivity in breast cancer [260]. This miRNA directly target the *RUNX2* that interacts with p53 and correlated with poor clinical outcome and resistance to anthracylines [260], but no studies have described its association to response after surgical approaches in any of the cancer. Therefore, it is interesting to assess whether miR-4454 and miR-4530 are targeting the same pathway or different pathways but likewise responsible of the poor prognosis and more aggressive phenotype of the cancer. In this current study, the low expression of miR-4454 in tumoral tissue and conversely, its high expression in CR from T0 to T6 could mirror the restoration of what might work as an oncosupressor in the liver. MiR-4454 was predicted to target *DLG5*, DLG5 is a scaffold protein belonging to membrane-associated guanylate kinase (MAGUK) family, mainly located at adherent junctions, where it plays a major role in the maintenance of epithelial polarity of cells as well as the migration potential of cells [272]. Its overexpression had been associated with malignancy, cell migration, invasiveness, and

resistance to chemotherapy in breast cancer setting [261]. Another interesting putative target is *BAG5*, an anti-apoptotic protein interacting with a variety of cell apoptosis and growth-related proteins, such as BCL-2 and Raf Kinase, that is associated with poor prognosis in pancreatic cancer [262]. However, as for mir-4454, none of these predicted targets have been studied in HCC, thus opening new perspectives for investigating their molecular role in HCC.

In the present study, the specificity of miR-4454, miR-4530 and miR-4443 as predictive biomarkers was proved for curative therapies. In patients receiving non-curative treatments such as TACE, we discovered that none of these miRNAs were differently expressed between CR and PR. On the contrary, miR-4492 was significantly associated with CR at T0. The value of this miR as a biomarker in TACE was by ROC curve analysis showing an AUC of 0.84 with a sensitivity of 84.6% and specificity of 71%, which is superior compared to the current biomarker, such as AFP, used for predicting responders after TACE [113,263,264]. The only study describing the role of miR-4492 in cancer comes from Lu *et al.* (2018) in colorectal cancer [265]. It was described that miR-4492 targets forkhead box K1 (*FOXK1)*, an oncogene that regulates proliferation and invasion in colorectal cancer cells [265]. Thus, miR-4492 itself might work as a tumor suppressor inhibiting oncogenes involved in drug resistance to TACE, explaining the low expression of miR-4492 in partial and non-responders and its low presence in tumoral tissue. Through an *in silico* prediction analysis, we identified KLK4 as one of the top scoring predicted target genes of miR-4492. Even though there are no record of evidence regarding its role in HCC, KLK4 had been associated to the expression of *VEGF* and regulation of angiogenesis in prostate cancer cell models [266]. Moreover, Tang *et al.* (2019) described the role of *KLK4* on cancer migration, invasion, and angiogenesis in gastric cancer [267]. However, the role of these targets should be validated in HCC and key mechanisms involved in the therapy response to TACE should be further investigated, especially considering angiogenesis pathways as a major player in determining the effectiveness of TACE [268].

Taken together, these results evidenced the distinctive profile of circulating miRNAs associates either with curative or non-curative treatments. This underlines the specificity on utilizing the appropriate miRNAs as predictive biomarker of TR for a specific type of therapy. This might be in line with the future goal to apply individualized treatment protocols to every single HCC patients.

At the time of admission, estimating the survival time for the patients would be very helpful for the clinician, in order to better stratify the patients and to assign priorities for treatments to some of them. Considering that reported OS for resection and TACE remains unsatisfactory [111,113], it is interesting to evaluate the potential of serum miRNAs as a predictors for OS in regards to pre-therapeutic stratification of HCC patients. In the present study, at T0, we observed that higher expression miR-4507, mir-4423-3p and mir-3185 were significantly associated with longer OS, in agreement with our previous results from microarray analysis. Indeed, from Kaplan-Meier Analysis, we observed significant differences in the OS of the patients having higher miR-4507 and miR-3185 expression before treatment compared to the ones with lower expression. These results are more striking when considering that patients with low expression of miR-3185 have comparable OS to patients that did not received any treatment. These results demonstrated the potential of miR-4507 and miR-3185 as novel circulating biomarker to predict OS before treatments, considering no study has ever analyzed the association of these miRs with any type of cancer, suggesting the novelty to further explore and validates its role in cancer pathways. Indeed, *in silico* target prediction analysis shown that ERCC1, one of prominent gene associated with cisplatin resistance and poor prognosis in HCC, as a possible target of miR-3185 [269,270]. In fact, Ryu *et al.* (2017) reported that cervical cancer patients with low expression of ERCC-1 had longer OS after treatment [271]. Thus, it is interested to validate this study in order to confirm the relation and role of the tumor-supressor role of miR-3185 to inhibit ERCC-1.

Our result from this task has sucessfully identified the potential of serum miRNAs as prognostic biomarker for HCC. Here we identified a panel of novel miRNAs that were never reported as a non-invasive biomarker candidate in HCC setting but proven to be associated with cancer pathways in other cancer. With this study, we also performed a longitudinal observation of the dynamic changes of our panel of miRNAs at three time points during HCC treatment. Our findings might have a clinical relevance considering that the current suggested biomarker for HCC, such as AFP, remains unsatisfactory. The dicriminatory potential for TR, DFS and OS of our miRNAs panel at T0, strengthen the relevance of miRNAs as circulating biomarkers supporting the idea of individualized treatment strategies based on risk prediction model to predict the outcomes for each type therapy. However, the present study has some limitations. It is essential to address whether the alteration of serum miRNAs is originated from tumor cells or attributed to a non-specific response to the presence of cancer and/or to

therapy itself. Even though we confirmed the presence of our miRNAs candidate in tumoral and distal HCC tissue, the very small sample size (n=10) cannot be a reliable evidence to derive certain conclusion. Hence, it is essential to increase the sample size and to include healthy tissue as a comparative control.

5.3. MiR-4454 targets DLG5 and inhibits the migration of HCC cells

Accumulating evidences suggested that miRNAs are important regulators in various cellular processes, including in the development of HCC. However, the precise role of various miRNAs in the development of HCC is still largely unclear. This includes also miR-4454, one of the circulating biomarkers we found associated to HCC prognosis. We decided to focus on miR-4454 based on the consistency of its differential expression related with the therpay response of HCC patients underwent curative treatments, and its consistent dysregulation in our paired tumoral and distal tissues from HCC patients. Moreover, from a clinical standpoint, confirming the potential of miR-4454 to predict response of curative treatments would significantly help clinician on determining patients that will respond to curative approach such a surgical resection and RFA. On the other hand, it will provide an early opportunity for clinician to decide another therapeutic approaches to patients that is ineligible of curative treatments, considering that most of patients at this group will have a much conserved liver function or early stages (0/A) [80,92].

According to the *in silico* target prediction analysis, DLG5, BAG5, and EIF4A2 were identified as putative targets of miR-4454. Moreover, the expression of the three target genes were upregulated in 90%, 80% and 60% of our paired tumoral tissue, respectively, fitting with the trend of downregulation of miR-4454 in these tissue samples. Indeed, based to the available literatures, only few studies have described the role of these genes in cancer setting, thus adding another novel element to explore the role of miR-4454 *in vitro*.

When validating these target candidates *in vitro* by performing a transient transfection of miR-4454 mimic to JHH6 cell line, we observed a clear downregulation for DLG5, but not for the other two candidates. Thus DLG5 was the only predicted putative target that was validated both in tumoral tissue and *in vitro*. In accordance with the role of DLG5, we observed an inhibition in cell migration after miR-4454 transfection. Thus we

hypothesized that miR-4454 inhibited the migration of HCC cells *in vitro* by targeting DLG5. However, more experiments are needed to support this hypothesis.

Despite the few available literatures that assign a major role in the maintenance of epithelial polarity of cells as well as the migration potential of cells to DLG5 [272], still conflicting data exists about this protein. Indeed, it is the loss of DLG5 that was associated with tumor development in HCC [273]. On the contrary, Saito *et al*. (2018) highlighted the fact that several proteins involved in cell polarity regulation, such as DLG5, might not only be abundantly expressed in normal cells as a fundamental property to generate cells structure, but might be frequently up-regulated in tumor cells being mislocalized from cell-cell junctions to a subcellular level, thus supporting intracellular asymmetry alterations in tumor cells that contribute to proliferation, apoptosis, and stress adaptation in cancer pathways [274]. Therefore, it is highly possible that DLG5 expression might also be upregulated in our cancer cells model and miR-4454 might acts as a tumor-supressor miRNA that targets and inhibits DLG5 on both mRNA and protein level. Indeed, explorations in Starbase and Human Protein Atlas Databases support our findings with a DLG5 up-regulated in tumor tissue. In addition, higher DLG5 expression was associated to lower survival probability. Interestingly, this prognostic data are in agreement with our miR-4454 results in serum,which associating the decrease of circulating miR-4454 to shorter overall survival.

5.4. Limitation and future perspective

The present study has some limitations. It is essential to address whether the alteration of serum miRNAs is originated from tumor cells or attributed to a non-specific response to the presence of cancer and/or to therapy itself. Moreover, there is a possibility that miRNA expression in serum can also have an opposite level of expression than in tissue or cells. Bai *et al.* reported that the great part of the miRNA, which was upregulated in HCC patient blood, was somehow downregulated in tumors and *vice-versa* [275]. Using HCC cell lines (HepG2 and Huh7), the group shown that cancer cells may selectively release miRNAs, thus opening a hypothesis that miRNA expression in cancer tissues and corresponding serum might only be partially the same, and cells might selectively decide which miRNA released into serum, even though the exact mechanism needs to be further explored [275]. Therefore, even though we confirmed the presence of our miRNAs candidate in tumoral and distal HCC

tissue, the very small sample size (n=10) cannot be a reliable evidence to derive an absolute conclusion. Hence, it is essential to increase the sample size and to include healthy tissue as a comparative control. On the other hand, to validate our miRNA panel as a reproducible diagnostic and predictive biomarker, there is an urgent need to assess their expression in a larger and heterogenous cohort with different etiologies.

Moreover, we have only assessed DLG5 as miR-4454 target and its possible functional role. Considering that we have only proved DLG5 expression in mRNA level, more experiments are needed to validate DLG5 as miR-4454 target. We also need to conduct more functional assay analysis to reveal miR-4454 role in other important cancer mechanism such as proliferation, necrosis, or apoptosis. Moreover, we aim to explore the role of the other miRNA biomarker candidates in HCC, since they are never or insufficiently described in literature.

From technical standpoint, since there are general lack of agreement in the methods for analyzing miRNA expression data, with this study we also identified and used a novel stable reference gene, such as miR-1280, that also must be validated in external cohorts. Lastly, to ensure the reproducibility of this results, a multi-center validation study needs to be conducted in a larger cohort considering that individual variability such as gender, race, or etiology of cancer might also influencing miRNA profile.

Chapter 6

Conclusion

In this project, we revealed the potential of several novel serum miRNAs as a non-invasive tools for early HCC detection and as prognostic predictors of HCC treatments. We assessed the role miR-3197 in HCV patients, before and during DAAs therapy, as potential biomarker for the prediction of the liver tumor occurrence in this setting. As a prognostic biomarker, we identified the potential of a novel miRNA panel to predict patients response before the initiation of therapy, specific foreither curative or non-curative treatments. MiR-4454, miR-4530, and miR-4443 were associated with curative treatments while miR-4492 was associated with non-curative treatments (TACE). We also described the potential of miR-4454 and miR-4530 as predictive biomarker for DFS and miR-4507 and miR-3185 as predictive biomarkers for OS. Moreover, we discovered the potential target genes of our panel of miRNAs that are associated to cancer survival, invasiveness, and resistance to treatment. Indeed, *in vitro* analysis shown that miR-4454 represses the migration of tumor cells by possibly targeting DLG5. Taken together, these results evidenced the distinctive profile of circulating miRNAs that can be applied in future clinical protocols, especially in high-risk population, that include prediction tools to the occurrence of HCC and the response to treatment, in corcondance to the idea of individualized treatment and to improve the survival of HCC patients.

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