

**ROLE OF MICRORNAS IN HEPATOCELLULAR CARCINOMA
DEVELOPMENT AND METASTASIS**

by

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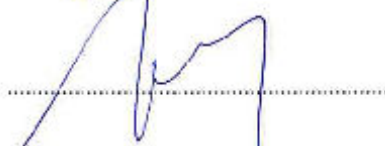
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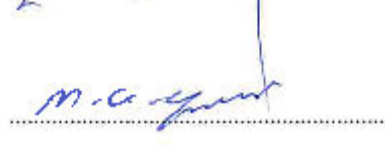
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ROLE OF MICRORNAS IN HEPATOCELLULAR CARCINOMA DEVELOPMENT AND METASTASIS

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ABSTRACT

Autophagy is a cellular stress response mechanism whose activation leads to the degradation of proteins as well as damaged organelles for maintaining cellular homeostasis. Autophagic capacity or alteration of its activity was found to relate with several pathologies, including cancer and metabolic diseases. Besides, microRNAs (miRNAs) have implicated in several fundamental biological processes such as cellular transformation, carcinogenesis, and even autophagy. Deregulation of both autophagy and miRNA pathways have found to contribute to the pathogenesis and progression of hepatocellular carcinoma (HCC), which is one of the mortal types of cancer. Deregulation of autophagy-regulating miRNAs addressed almost all stages in HCC tumorigenesis from manifestation of low-grade dysplastic nodules to advanced disease. Thus, manipulation of autophagy by miRNAs might provide novel targeted therapy options for HCC.

HEPATOSSELÜLER KARSİNOMA GELİŞİM VE METASTAZINDA MİKRORNALARIN ROLÜ

Yunus AKKOÇ

Moleküler Biyoloji, Genetik ve Biyomühendislik, Doktora Tezi, Temmuz 2019

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Anahtar Kelimeler: Hepatoselüler karsinoma, microRNA, *MIR376B*, otofaji, tümörögenez

ÖZET

Otofaji, proteinlerin ve hatta hasarlı organellerin temizlenmesinde rol alarak hücrel homeostazı sağlayan bir stress yanıt mekanizmasıdır. Otofaji kapasitesi veya aktivitesindeki değişiklikler metabolik hastalıklar ve kanser dahil olmak üzere birçok patoloji ile ilişkilendirilmiştir. Buna ek olarak, mikroRNAların da hücrel değişim, karsinogenez ve hatta otofaji gibi temel hücrel olayları düzenlediği bildirilmiştir. Hem otofaji hemde miRNA yollarında meydana gelen bozukluklarının en ölümcül kanser türlerinden biri olan hepatoselüler karsinoma (HCC) patogenezi ve seyirine katkıda bulunduğu ortaya çıkarılmıştır. MiRNA regülasyonlarındaki değişikliklere düşük dereceli displastik nodüllerin oluşumundan ileri HCC ye kadar hepatokarsinogenezin hemen hemen tüm aşamalarında rastlanmaktadır. Bu nedenle, miRNAlar tarafından sağlanan otofajik düzenlenmenin bize HCC için yenilikçi ve hedefli tedavi seçenekleri sağlayabileceği düşünülmektedir.

“To those who let the future tell the truth, and evaluate each one according to their work and accomplishments. The present is theirs; the future, for which I have really worked, is mine.”



To my family

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“Life is not easy for any of us, but what of that? We must have perseverance and above all, confidence in ourselves. We must believe that we are gifted in something and that this thing, at whatever cost, must be attained.” As Marie Curie said, it was not that easy, yet I always believed to the power of knowledge and my endless dedication during the Ph.D. period. However, I would never be able to finalize my work without the support and guidance of the following people. Therefore I would like to express my most profound appreciation to them.

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I try to understand and search for the meaning of life by science. However, none of the known equations, reactions, or biological phenomena have thoroughly convinced me. Then I realized the final part of the puzzle, and I addressed a new approach to focus on little miracles out of Virginia Wolf's words, *"What is the meaning of life? That was all a simple question; one that tended to close in on one with years, the great revelation had never come. The great revelation perhaps never did come. Instead, there were little daily miracles, illuminations, matches struck unexpectedly in the dark; here was one."* That is why I also would like to thank my family and express my gratitude. They endlessly and unconditionally support me; they were my real little miracles in all along this process.

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LIST OF ABBREVIATIONS

microRNAs	MiRNAs
miRNA response element	MRE
RNA Induced Silencing Complex	RISC
Autophagy related genes	ATG
Hepatocellularcarcinoma	HCC
Transarterial chemoembolization	TACE
Hepatitis B Virus	HBV
Hepatitis C Virus	HCV
Hepatitis C Virus	HDV
Non-Alcoholic Fatty Liver Disease	NAFLD
Non-Alcoholic Fatty Liver	NAFL
Relax circular DNA	rcDNA
Closed circular DNA	cccDNA
Nucleotide analogues	NAs
HBV Surface Antigen	HbsAg
Human Immunodeficiency Virus	HIV
Hepatitis B Virus X protein	HBx
Sustained Virologic Response	SVR
Direct-acting Antivirals	DAAs
Reactive Oxygen Species	ROS
Free fatty acids	FFAs
Senescence-associated secretory phenotype	SASP
Alcoholic liver disease	ALD
Aflatoxin B1	AFB1
Alpha-fetoprotein	AFP
Portal hypertension	PHT
Barcelona Clinic Liver Cancer	BCLC
Portal vein thrombosis	PVT

Maximum tumor diameter	MTD
Low-grade dysplastic nodules	LGDNs
High-grade dysplastic nodules	HGDNs
Tyrosine kinase inhibitor	TKI
Overall survival	OS
Dimethyl sulfoxide	DMSO
2',7' –dichlorofluorescein diacetate	DCFDA
Wild-type	WT
Quantitative reverse transcriptase PCR	qRT-PCR
Reverse transcriptase PCR	RT-PCR



1 INTRODUCTION

Cancer is an emerging epidemic, which is one of the leading cause of death after cardiac diseases. According to global cancer agency data, each year, almost 2 million new cases have been diagnosed, and numbers are getting higher each every following year [1]. Understanding this pathology is quite crucial to develop new treatment strategies. In the last decade, there have been many explorations came out about dissecting the molecular biology of cancer. In parallel, technological development also helps us a lot in terms of screening and early prognosis of the burden.

Cancer cells are considering as a type of transformed cells. Thus, studies showed that they exert almost complete distinct pattern of altered cellular signaling in comparison to their healthy counterparts. Therefore, new approaches focused on exploring the changes in the cellular signaling pathways. Autophagy is one of the main types of the cellular degradation process, and as and cellular stress response mechanism, it is conserved from yeast to man [2]. Under basal conditions, autophagy naturally being active and help cells to maintain their cellular homeostasis through regulating the degradation of expired proteins and damaged organelles and fuels cells with new cellular building blocks [3]. However, in as much as other cancer-related pathways, autophagic alterations have been associated with the pathology of cancers, including hepatocellular carcinoma (HCC). HCC is one of the mortal types of cancer, and its incidence is rising, especially in developing countries upon dietary changes. Etiology of the disease have studied widely, and there are several facts behind this burden. For instance, viral infections, environmental toxin exposure, metabolic syndrome documented as one of the main factors of this deadly disease. Not surprisingly, modification of autophagic activity addressed in many studies to treat HCC.

MicroRNAs are one of the non-coding RNAs, and they act as a post-translational modifier in cells. MiRNAs have associated with the regulation of many cellular processes, including autophagy and cell death. More than that, as they are one of the cellular fate controlling switches, their deregulations implicated in various pathologies including HCC. Therefore, the identification of both autophagy and miRNA deregulations in HCC may lead to the development of novel therapeutics in combat with this deadly disease.

1.1 Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma (HCC), is one of the primary types of liver cancer and is the sixth common cancer types worldwide (**Figure 1.1**) [1, 4]. It may not be the most diagnosed cancer, yet HCC has one the second-highest mortality rates among other types of cancers in men (**Figure 1.2**). Recently released cancer data showed that there were approximately 840,000 new HCC cases have diagnosed, and estimations suggest 61 % increase in the HCC cases in the year 2040 [1]. Besides, HCC prevalence has not evenly distributed throughout the world, the vast majority of the HCC cases occur in the notably less developed region of the world, such as Eastern and South-Eastern Asia and sub-Saharan Africa (**Figure 1.3**) [1, 4].

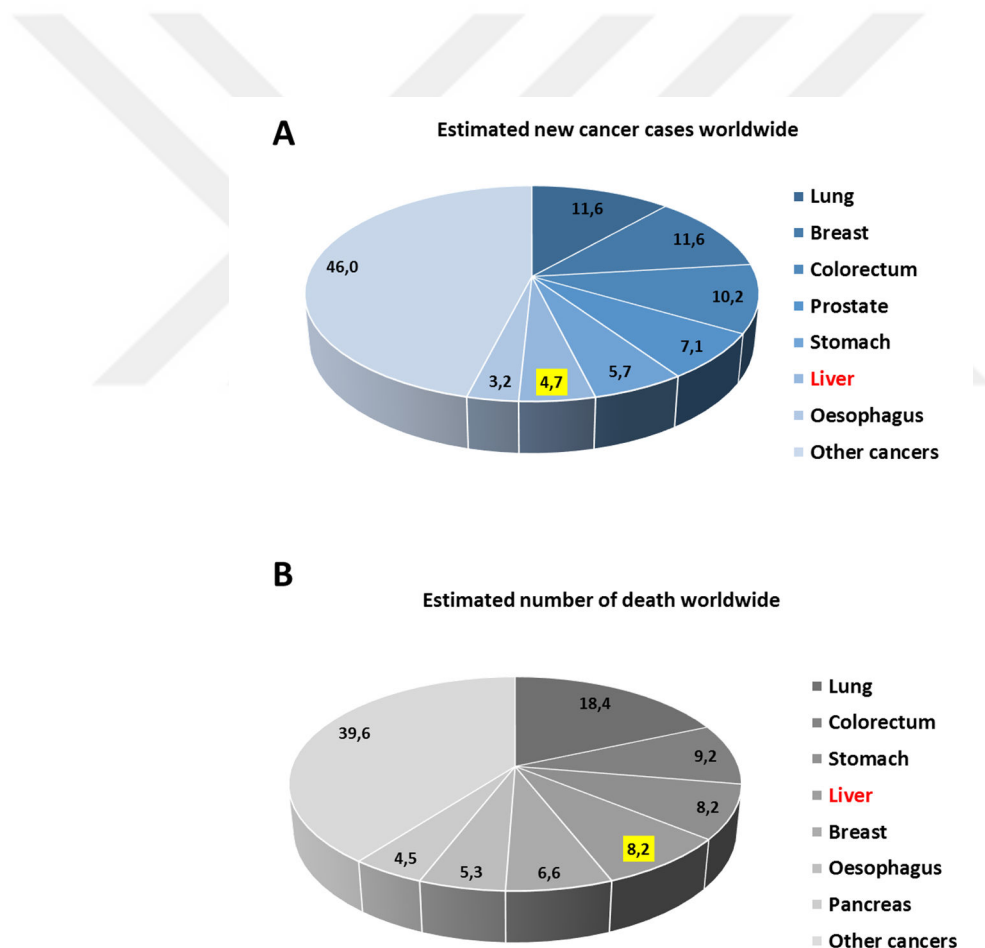


Figure 1. 1 Schematic representation of A) Estimated cancer incidence B) Cancer-related death worldwide. In each pie chart, liver cancer percentage in total cases were colored and specified. Pie charts adapted from GLOBOCAN, Global Cancer Observatory, released 2018 data (<https://gco.iarc.fr/>).

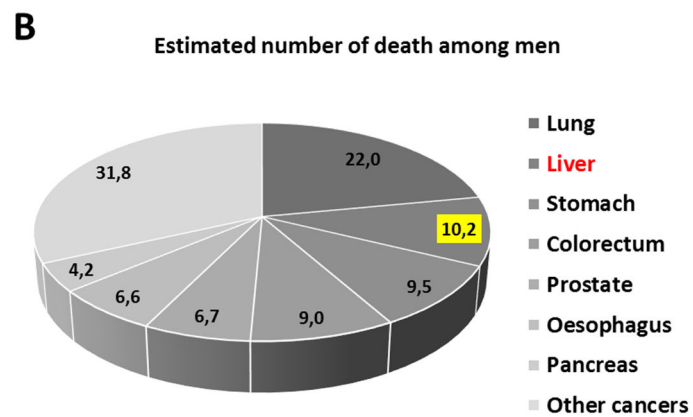
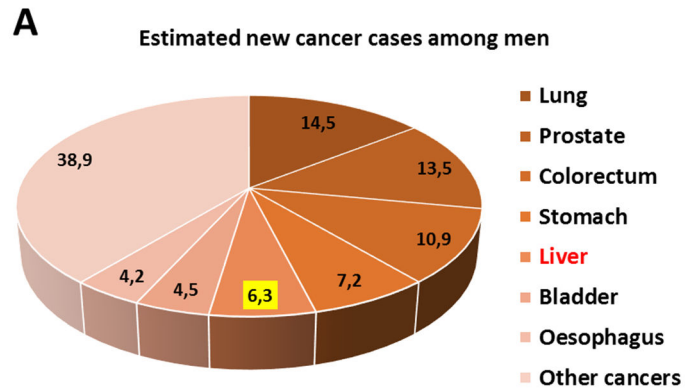


Figure 1. 2 Schematic representation of A) Estimated cancer incidence B) Cancer-related death among men. In each pie chart, liver cancer percentage in total cases were colored and specified. Pie charts adapted from GLOBOCAN, Global Cancer Observatory, released 2018 data (<https://gco.iarc.fr/>).

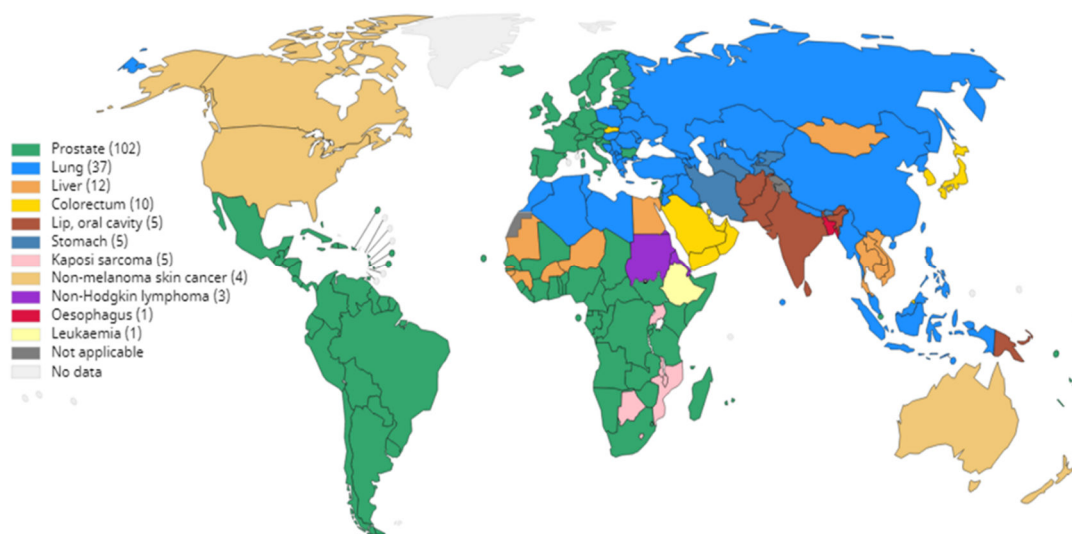


Figure 1. 3 Representation of an estimated new cancer cases by top cancer cases per country among men in 2018. World Map adapted from GLOBOCAN, Global Cancer Observatory, released 2018 data (<https://gco.iarc.fr/>).

Despite the gender, HCC incidence has also fluctuated among different ethnicity. According to United States-based data the ratio of HCC, per 100.000 age-standardized rates (ASRs) of HCC at all ages and gender, is almost 13.1 % in Asians and Pacific Islanders yet, it is comparatively small for instance 4.5 % in Caucasian ethnic background people [5]. In as much as the ratio of increase HCC incidence, etiology of the disease is also varying in different ethnicity. HCV infections seem to be responsible for the HCC development, while Hispanic ethnic background people developed HCC mostly because of NAFLD [6-8]. Of note, HCC incidence and etiology may also vary in the same country. For instance, according to the study revealed in Turkey showed that HCC etiology were different even from the cities that reside in the same geographical region [9]. The reason for the interethnic variability may arise from various genetic alterations or population dynamics such as average age and or gender distribution. Hence intra-ethnic differences more likely to be associated with the difference and the exposure time of major risk factors.

Almost 70-90 % of the HCC developed upon cirrhotic background, and the scenario is more dramatic in Western countries comparing the rest of the world [10, 11]. Chronic inflammation and subsequent liver damage provoke permanent hepatic injury were then eventually developed cirrhosis [10, 12]. Upon injury, liver fibrosis occurred at first glance, which considered as a classical healing response in the wounded tissue. Concomitantly, cirrhosis has classified as a final stage of the liver fibrosis following with distorted liver parenchyma and altered blood flow [13]. In line with this, several secondary complications may arouse, including portal hypertension and ascites accumulation and further provoke loss of liver function [14-16]. HCC has developed at any stage of cirrhosis.

Despite the tremendous therapeutic development against to cure and manage liver diseases over the past era, cirrhosis still represents a significant portion of liver-based health burden [17]. Liver cirrhosis and its secondary complications are also affecting the prognosis of HCC patients and even treatment modalities[10, 18, 19]. For instance, according to the level of cirrhosis, standard approaches for HCC treatment including resection and transarterial chemoembolization (TACE) may cause unexpected complications and injured non-cancerous liver which further affects the healthy liver function [20, 21].

1.1.1 Risk Factors

HCC, mainly occurring at the background of inflammation and chronic liver diseases such as viral infections, fibrosis, and cirrhosis. HCC, as one of the primary liver diseases, may provoke by hepatotropic virus infections such as hepatitis B, C, and D viruses (HBV, HCV, HDV respectively), alcohol abuse and environmental toxin exposure. More than that increased number of cases have also demonstrated that non-alcoholic fatty-liver disease (NAFLD), obesity and metabolic syndrome are also crucial shareholders in the risk factors of development of HCC [10, 12].

1.1.1.1 HBV Infection

HBV is a hepatotropic virus, classified as a member of a family of small, enveloped DNA viruses called hepadnaviruses. The genome of HBV is circular, and its replication happens via an RNA intermediate [22]. The HBV genome consists of two asymmetric DNA strands, which forms a partially double-stranded relaxed circular DNA (rcDNA) structure[23, 24]. Upon HBV infection, the disintegration of nucleocapsid leads to the release of rcDNA, which then transported to the nucleus and converted to the closed circular DNA (cccDNA). cccDNA found to be a form of freestanding episomal conformation and have an interaction with host nuclear proteins, including histones. Importantly, in the way of cccDNA, DNA replication resides inhibited. Therefore, it creates resistance against nucleotide analogs (NAs). Although clearance of HBV surface antigen (HBsAg) has achieved following antiviral therapy, cccDNA may still exist in the host nucleus, which further may cause recurrence [25, 26].

More than 350 million individuals are suffering from chronic HBV infection in the world [27]. Thereby, chronic HBV infection is considered the leading risk factor of HCC development [28]. For instance, chronic HBV infection assessed as a prominent risk factor of HCC in particular at Southeast Asia and sub-Saharan Africa. It is generally transmitted from mother to the children during birth, where it is highly endemic; however, parental and sexual contact of contaminated blood is the main reason in developed countries.

In addition to its single effect, it may also play an essential role with other risk factors, including coinfection with HDV, aflatoxin B1 exposure [29-31]. Like HDV coinfection, HBV co-infection with Human immunodeficiency virus (HIV) also increase the risk of HCC development [32]. For instance, more than 70 % of HCC developed upon HBV infection in Africa. Besides, potential exposure of aflatoxin in this region is also increasing the risk and led to develop HCC in early ages which synergistic effect declared almost had 60 times more chance to develop HCC than healthy individuals [33, 34]. In addition to the synergistic effect of both factors, certain HBV strains, including genotype C in Asia and genotype F in Alaska, and mutations in the HBV genome associated with increased HCC risk [35].

HBV surface antigen (HBsAg) and serum HBV DNA level associated with HCC patients [36]. Besides, HBV DNA integration in hepatocytes is also another vital sign of the high-risk group. Among HBsAg carriers, HBV DNA integrated hepatocytes positive individuals exert 100 times more risk than negative one [37]. Of note, several factors including age, gender, and duration of HBV infection, alter the risk of developing HBV-related HCC. Upon HBV infection and HBV DNA integration, it causes severe cellular, inflammation, and subsequent increase in proliferation [35]. Although HBV integration was primarily assumed to be random, there are several reports showed HBV DNA integrates some specific places in the host genome. HBV DNA integration into the host genome caused a unique feature which drives the activation of oncogenic signals and hepatocarcinogenesis even under non-cirrhotic microenvironment [26]. In general HBV infection drives hepatocarcinogenesis such as inducing immune-mediated hepatic inflammation, ROS induction, hepatitis B x protein (HBx) and HBs proteins involving virus-specific mechanisms, insertional mutagenesis with the integration of HBV DNA into the host genome, epigenetic modification such as deregulation of miRNAs and genomic methylation status. All these events and prolonged inflammation led to liver injury and hepatic fibrosis, which cause several architectural changes in liver and facilitate the development of HCC over time [37].

Primary, prevention from HBV achieved by vaccination programs. In some particular regions, HCC incidence determined significantly lower among vaccinated children compared to unvaccinated cohorts [38]. In addition to vaccination, antiviral therapies as a secondary approach have also utilized, and interferon-based regimes succeed to decrease both cirrhosis and HCC development [39].

More than interferon-based regimes, NAs are also used and showed reduction of HCC development in Taiwanese and Japanese cohorts [40-42].

1.1.1.2 HCV Infection

HCV is a single-stranded RNA virus with positive polarity and classified as an enveloped RNA virus under the *Flaviviridae* family [43]. HCV genome encodes a large protein consist of 3000 amino acids which then undergoes proteolysis to give rise two sets of proteins; structural proteins (Envelop E1, E2, and Core) and nonstructural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [44].

HCV is the second leading cause of HCC behind HBV infection worldwide. Approximately 170 million people are becoming chronic carriers of HCV [5]. According to o global data, the highest amount of infection ratio reported in Eygpt (18 %) and in Mongolia (10 %) [45, 46]. In contrary to HBV, HCV infected cohort in the high risk of developing cirrhosis. Moreover, contrary to HBV, HCV infected cohort have developed HCC in later years of their life [47]. HCV and HBV coinfection reported developing the risk of HCC more than 4 fold in comparison to single-agent [48]. HCV viral infection pledged for the enormous increase of HCC incidence one of the major spread of HCV infection took place in Japan [49]. Unlike other Asian countries which exert a high ratio of HCC, such as China, HCV is the dominant viral risk factor of HCC in Japan.

Unlike HBV, almost all HCV-related hepatocarcinogenesis arise due to cirrhosis or chronic inflammation. HCV is a non-cytopathic virus, and mechanism of action in HCC development related to HCV infection differ from HBV, which infection provokes hepatic injury by immune-mediated reaction-cascade. Therefore, HCV suggested as an indirect carcinogenic agent by induced inflammatory and necrotic lesions. HCV-related HCC has associated with several cellular phenomena, including cell death, regeneration, inflammation, oxidative stress, and steatosis [50-52]. Of note, a bunch of studies showed that viral proteins, including core proteins, alter various cellular functions including apoptosis, and suppresses p53 activity which leads to decide the fate of HCC development [53-55]. However, rather than viral proteins and factors, environmental and host-dependent factors seems to be more critical to the determination of cirrhosis [56].

HCC incidence significantly reduced in patients who are eligible to receive and tolerate to antiviral therapies and exert sustained virologic response (SVR) [57]. Estimated HCC incidence increase even though the fact that improved SVR rate has ensured by direct-acting antivirals (DAAs) [58, 59]. Unfortunately, high DAA costs, lack of comprehensive HCV screening, drug abusers, and a significant proportion of unaware HCV carriers block the reduction of HCV-related HCC ratio [58, 60]. However, DAA treatment to prevent re-infection after transplantation seems to be cost-effective according to the level of disease burden [61]. Either interferon-based SVR or DAA and interferon combined strategies showed a promising reduction in HCC incidence [62, 63]. High viral genetic variability of HCV decreases the chance of developing a prophylactic HCV vaccine as primary prevention [64]. Targeting host genes/proteins such as viral entry factors have significant potential as an alternative treatment strategy.

1.1.1.3 Diabetes and Obesity

In the last decade, both obesity and Type-2 diabetes mellitus have reached epidemic proportions and severely threaten public health.

Serum insulin and insulin-like growth factor-1 (IGF-1) elevated in diabetic patients. Surprisingly, both factors found to be associated with the increasing incidence of HCC [65-67]. Even though the fact of how diabetes support HCC development has not fully elucidated, studies showed that diabetes-assisted inflammatory cytokine increase, such as IL-6 and TNF- α found to trigger intracellular ROS accumulation and genotoxic stress [68]. Subsequently, diabetes-induced inflammatory cascade further facilitates the hepatic damage and provokes hepatic fibrosis and even cirrhosis, which prepare the hepatocarcinogenic environment in the liver.

Like diabetes, a strong correlation between obesity and cancer also documented in many studies. Although primarily obesity called as a risk factor of cardiovascular disease, now it also suggested as one of the crucial risk factors for hepatic pathologies, including NAFLD and HCC [69, 70]. Concomitantly, several studies showed that obese individuals have a high risk of developing HCC almost two-fold more than non-obese cohort [71, 72].

Obesity-related HCC underlined in several biological phenomena. Insulin resistance and its subsequent inflammatory response found to be associated with obesity-related HCC [73]. In one hand, insulin resistance increases the hepatic fat accumulation via increased delivery of free fatty acids (FFAs) and the effect of hyperinsulinemia [74]. On the other hand, obesity also linked with a low-grade inflammation upon increased production of cytokines, including IL-6 and TNF-alpha. Reduced levels of adiponectin, hepatic lipid accumulation where can be used as fuel of aggressive cancer growth and lipotoxicity also classified as an essential determinant in obesity-related HCC [68, 75, 76]. Recently, intestinal microbiota (dysbiosis) changes following dysregulation of inflammatory cytokines are also linked with obesity-related hepatocarcinogenesis [77-79]

1.1.1.4 Non-alcoholic fatty liver (NAFL), Non-alcoholic fatty liver disease (NAFLD) and Non-alcoholic steatohepatitis (NASH)

Non-alcoholic fatty liver (NAFL) is associated with steatosis where the triglycerides accumulated in hepatocytes, yet there is no, or less damage and inflammation occur in hepatocytes. In contrast, NAFLD is a chronic liver disease commonly characterized by the excessive cytoplasmic retention of triglyceride, inflammation, and ballooning of hepatocytes, which further alter the architecture of the liver. NASH is a more aggressive type of NAFLD and often accompanied by a high level of cirrhosis (**Figure 1. 4**)

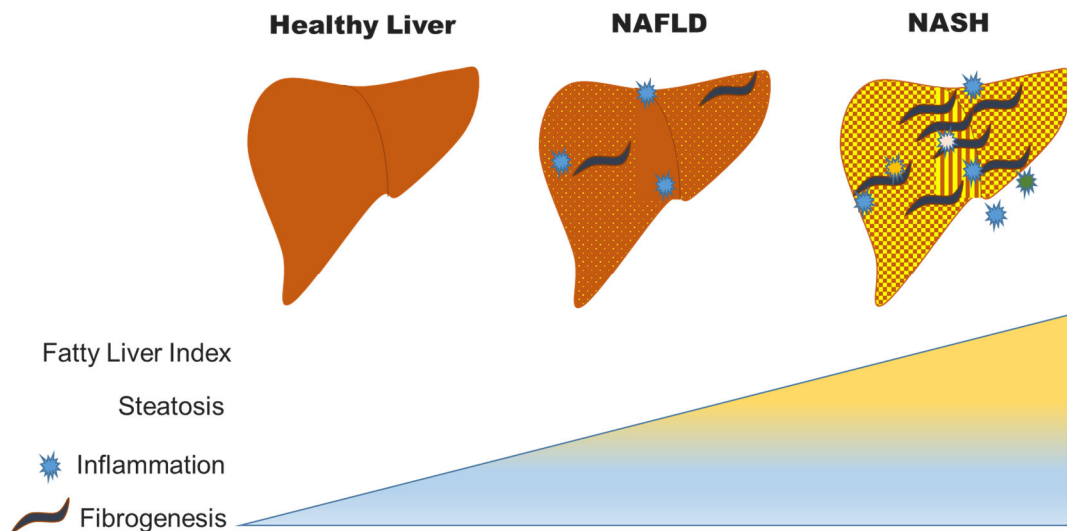


Figure 1. 4 Progression of NAFLD and NASH. Especially triglycerides accumulated in hepatocytes, which increased fatty liver index. Following lipotoxicity upon steatosis, pro-inflammatory cytokine release provokes the inflammation and caused liver damage. Fibrogenesis and stellate cell activation primed. Chronic liver damage leads to the cirrhosis and function loss of the liver. In clinical routine as a non-invasive method, abdominal ultrasonography detects fatty liver. Magnetic resonance imaging also helps to decipher stiffness and elasticity of the liver, which fibrosis and cirrhosis severely alter.

Non-alcoholic fatty liver disease (NAFLD)-related HCC incidence increased rapidly, and it is more prominent in developed countries [80]. Non-alcoholic steatohepatitis (NASH) is another liver disease which reflects a more severe phenotype in comparison with NAFLD and leads to the development of cirrhosis. Unfortunately, almost 60 % of the biopsied NAFLD patients have NASH[70]. NAFLD misdiagnosis and categorization errors of such as uncertainty about NASH-related cirrhosis and cryptogenic cirrhosis obscured the increased risk of NAFLD as an important HCC risk factor. More than that, in many regions, NAFLD, NASH, or even metabolic syndrome are not quantified. Although the risk of HCC due to NAFLD was lower than that due to chronic HCV infection, the high prevalence of NAFLD and its estimated increase become one of the major concern in the field of HCC research.

Dietary regimes, caloric consumption, and lifestyle are one of the major determinants in both liver pathologies. However, some genomic alterations in genes which often related to lipid metabolism were also associated with these liver diseases [81, 82]. Of note, both pathologies found to accompanied with other diseases obesity, Type 2 diabetes, dyslipidemia, and hypertension, which all considered as metabolic syndrome and exerted a synergistic effect of HCC development [70, 83].

Pathogenesis of steatohepatitis modeled as the two-hit hypothesis. Primarily, hepatic lipid accumulation (steatosis) occur than oxidative stress increased due to lipotoxicity and subsequent inflammation [84, 85]. Mechanism of NAFLD and NASH-related HCC still deciphering. Studies showed that upon lipid accumulation in hepatocytes caused lipotoxicity and provoke chronic damage through triggering ROS production, ER stress, inflammation, and eventually apoptotic or necrotic cell death. Prolonged inflammation also facilitates the immune cell filtration, fibrogenesis, and activation of hepatic progenitor cells [86-88]. The hepatic stellate cell is one of the scarring tissue components and often increased upon liver damage. Bacterial metabolite (deoxycholic acid)-induced senescence-associated secretory phenotype (SASP) facilitate the activation of hepatic stellate cells, and it linked with NAFLD [79]. Immunosuppression also addressed as a critical player in NAFLD-related HCC. Hepatocyte derived linoleic acids alleviate the number of anti-tumor CD4 positive T cells and support immune-surveillance of HCC [89].

1.1.1.5 Other Factors

As its function liver, has an essential role in metabolic processes such as fat, carbohydrates and protein metabolisms and also clearance of the toxins such as alcohol or xenobiotic by-products. The liver can process, stored, altered, detoxified to these substances and pass them to the bloodstream or released them into the bowel for elimination.

Alcohol is one of these substances which processed by the liver. Alcohol abuse leads to severe liver damage and eventually induce cirrhosis. Risk is particularly high in northern and central Europe, where its consumption is top [90]. Alcoholic liver disease (ALD) and ALD-related cirrhosis differ from NAFLD. Triglyceride-induce degeneration of liver cells occurs to a higher degree in NAFLD in comparison to ALD. In contrast, inflammatory cell infiltration is more pronounced in ALD than in NAFLD [91]. Furthermore, fibrosis around the venous or perivenular area, and phlebosclerosis are more common in ALD than in NAFLD. Of note, cirrhosis may not be the only way to provoke HCC in NAFLD; however, in ALD, cirrhosis is a more prominent process which triggers to HCC development [92]. Many studies address the positive correlation between alcohol abuse and HCC [93].

Excessive alcohol intake predominantly increased the ROS production and liver damage following inflammation and cirrhosis. Moreover, alcohol increases the permeability of the gut wall and allow to transfer of pathogens called endotoxins into the bloodstream [94]. Endotoxins accumulation in the liver caused the accumulation of Kupffer cells, which are liver macrophages, and these also accelerate the inflammation and facilitate the loss of liver function too [95, 96].

Aflatoxin B1 (AFB1) is one of the well-studied mycotoxin produced by the *Aspergillus* fungus. Staple crops, including cereals, maize, and tree nuts, are susceptible to aflatoxin contamination. High humidity and un-optimized storage conditions prepare a favorable atmosphere where fungus can vegetate. Mechanism of action of AFB1 start after ingestion where it metabolites to an active intermediate called AFB1-exo-8,9-epoxide. Active compounds can bind DNA and block DNA synthesis, induce mutation on tumor suppressor gene p53, 249Ser [97]. AFB1 exposure monitored by the analysis of metabolites in patient urine, AFB1-albumin adducts in serum, and AFB1-induced mutations in affected tissue [98, 99]. Therefore, AFB1 identified one of the significant risk factors to increase the risk of hepatocarcinogenesis. The meta-analysis revealed out that AFB1 exposure increases the risk for HCC [100, 101]. AFB1 urine metabolites shown to increase the risk of HCC 4 fold, whereas HBV infection was increased 7 fold in China. Moreover, in the case of both condition carriers, the risk for HCC increased by almost 60 fold [102]. According to global data, nearly a quarter of all agricultural products estimated to be contaminated by aflatoxin. Unfortunately, aflatoxin intake peaked where the regions showed HBV endemic like southeastern Asia and sub-Saharan Africa. Several initiations have been conducted to reduce the level of aflatoxin exposure, including switching maize to another less aflatoxin susceptible crop, increasing public awareness, and strict governmental regulations [103]. In line with this, HCC incidence has been declined notably with birth cohort where attending governmental neonatal HBV vaccination in China [104]. In parallel, political changes in the regulation of changing agriculture of maize to rice had also declined the risk for HCC in China and Taiwan [103, 105].

1.1.2 Prognosis and Staging of HCC

Staging of cancer aims to stratify patient to understand their overall survival (OS) before treatment, accessibility to a specific type of treatment option, and further help to create an objective determinant for the research. In terms of HCC, staging is more complicated than many other solid tumors due to the fact of HCC development on chronic liver diseases, including cirrhosis, which severely affect the patient OS. Thus, the general health of the liver beside HCC is also one of the critical criteria in HCC staging [106, 107]. Therefore, HCC prognosis affected by several factors: Patient-derived factors including age and performance status; tumor burden including size, number of tumors and vascular invasion status; serum alpha-fetoprotein (AFP) level, liver reservoir according to Child-Pugh score, portal hypertension (PHT), platelet count, genetic factors, etc. [53, 108-111]. Almost more than 10 different staging classification proposed for prognosis evaluation [112, 113]. The physician needs to be guided correctly, approaching HCC for better survival and treatment selection. Thus, the choice of the best staging classification is crucial.

TNM generally measure according to the primary tumor size (T), presence and absence of tumor in the regional lymph node (N), and presence and absence of metastatic spread beyond the lymph nodes (M). TNM can either assessed before a treatment called 'clinical staging' or after surgery called 'pathological staging'[114]. However, both staging has its own obstacles. Clinical staging may misevaluate the HCC due to the fact of background liver diseases, including cirrhosis. Although pathological staging proposed for better assessment in comparison to clinical staging, yet a limited group of HCC patient undergoes surgical therapy. Most of the HCC patients are not suitable for surgical approaches either because of tumor extensions or poor liver reservoir. Thus, better staging classifications raised upon other criteria.

Barcelona Clinic Liver Cancer (BCLC) staging classification was proposed by Llovet and colleagues in 199 [112]. HCC follow-up data from patients who have non-resectable and non-transplantable play a crucial role in the development of BCLC [115]. Performance status, constitutional syndrome, vascular invasion, and extrahepatic spread identified as independent risk factors for mortality in BCLC.

In this system, patients can sub-grouped based on their tumor stage (tumor size, number of nodules, and presence of portal vein thrombosis (PVT)), liver function (Child-Pugh score, PHT), bilirubin level, physical status (performance status test) and cancer-related symptoms.

In line with these 4 different categories were created A, early; B, intermediate; C, advanced; D, end-stage disease. Of note, BCLC is also unique because it is the only system that provides treatment recommendations for each different categories. Both the European Association for the Study of the Liver (EASL) [116] and the American Association for the Study of Liver Diseases (AASLD) [117] endorsed BCLC staging classification for western patients. Yet, the prognostic implication of Barcelona Clinic Liver Cancer (BCLC) sub-stages in Asian patients with hepatocellular carcinoma (HCC) is still obscure.

HCC is a highly heterogenic disease with a lot of different risk factors which makes an assessment of the patients very complicated. Thus, with all these parameters and staging system needs to be updated and provide a common language to physicians for better treatment.

1.1.3 HCC in Turkey

HCC, as a complex disease, needs to be elaborately dissected according to region, risk factors, staging including liver and tumor factors, and extrahepatic conditions for better therapeutic approaches. Therefore, based on all these factors, a large scale study released from Turkey documented the fact of HCC in the region. Understanding the disease in Turkey is very important because it reflects both Mediterranean, Asian, and European characteristics at once. Maximum tumor diameter (MTD), portal vein thrombosis, and α -fetoprotein (AFP) levels have focused on this multicentric study [9]. Data obtained prospectively from 1,332 patients including clinical monitoring data, tumor parameters such as size, number of nodules, presence or absence of PVT, plasma AFP level, complete blood count, liver function tests including transaminases, albumin, and total bilirubin and also patient demographics.

Results reflect that HCC predominantly occurs in male, and the percentage is almost 82 %. In addition, as underlying liver disease, cirrhosis seen virtually in 82 %, which has a massive portion in these patients. In terms of etiological factors, HBV infection is the top with and 61 % ratio following HCV with a 21 %. Tumor burden is also quite crucial in the decision of treatment approach, and in this cohort, the mean MTD calculated as 5.89 cm. Serum AFP level manifested as 5,6 IU/ml. Importantly, serum AFP level of 42 % of patients seen in the typical laboratory values. In addition to AFP, other serological factors like albumin was also low, 3,9 g/dL and total bilirubin elevated at 2,96 mg/Dl [9].

In addition to these observations, they also figure out the correlation between the MTD and platelet counts. Platelet counts increased correlated with increased MTD. Platelet counts seem to classify HCC in Turkish patients' phenotype with better liver function but advanced HCC. More than that, patients were further evaluated according to the presence or absence of PVT. Data showed that PVT positive patients exert significantly large tumors with high platelet levels, AFP, C-reactive proteins, and elevated total bilirubin. The patient who has a high level of AFP also manifest high MTD and present PVT [9].

As another approach, data also evaluated according to different geographic groups from Turkey. Surprisingly, both MTD and PVT showed significant differences between these regions. Primarily fluctuations were found in cirrhotic patients among those regions. Viral infections identified considerably differ between analyzed areas in Turkey. HBV infection is almost 74 % in Diyarbakır. However, it is nearly 40 % in Hatay. In contrast, Hatay has the top HCV infections, around 31 % in comparison to Diyarbakır 8,37 % and Mardin 7,81 %, respectively. Of note, there is an increase documented for HDV infection in Diyarbakır approximately 17 %, yet it is below 10 % elsewhere [9].

1.1.4 Molecular Biology of HCC

Hepatocarcinogenesis is a complex process which requires many molecular alterations and several different pathways. HCC often occur upon cirrhosis and progression of the disease tightly correlated with the etiology of the underlying liver disease. HCC development follows several consecutive events. Pre-cancerous cirrhotic nodules followed by low-grade dysplasia called low-grade dysplastic nodules (LGDNs). After that, it subsequently transformed into high-grade dysplastic nodules (HGDNs) that can further develop into early-stage HCC and progress into more advanced HCC (**Figure 1.5**). Malignant transformation of HCC can originate from various liver resident cell types [118].

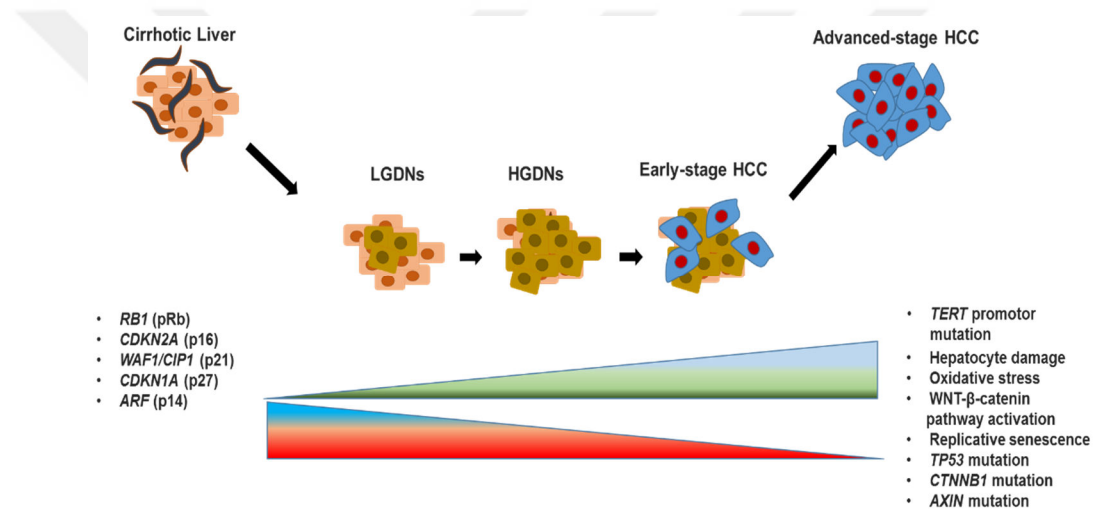


Figure 1. 5 Malignant transformation of hepatocytes and HCC progression. Molecular alterations and major molecular defects that observed early-stage hepatocarcinogenesis and mutation accumulation at a later stage of HCC.

Many somatic mutations addressed to accumulate in the progression of HCC. Mutations contribute to tumor evolution at any stages from initiation to metastasis by altering various cellular phenomena [119]. All genetic and epigenetic alterations eventually create a unique type of tumor which supports the also refer the tumor-heterogeneity in HCC [120, 121]. To discriminate early-stage HCC from the pre-cancerous nodules (LGDNs and HGDNs), various molecular markers identified which often called as early step markers in the context of cirrhosis [122-124].

Several mechanisms, including WNT- β -catenin pathway activation, re-expression of fetal genes, deregulation of protein-folding machinery, and the response to oxidative stress are often associated with the early events to malignant transformation of hepatocytes.

Alterations in the genes involved in telomere maintenance and telomerase complex are one of the core facilitators to the initiation and promotion of HCC underlying cirrhosis [125, 126]. For instance, telomerase RNA component (*Terc*) deficient mice exert short telomeres and developed cirrhosis followed by HCC [125]. Mutation in *TERT*, which encodes the telomerase reverse transcriptase, are also associated with an increased risk of cirrhosis in human [127, 128]. In contrary, a progression of HCC requires telomerase reactivation, which allows the uncontrolled hepatocyte growths [125, 129]. *TERT* is typically not expressed in hepatocytes; however, it re-expressed during early hepatocarcinogenesis in LGDNs and HGDNs [130, 131]. Studies showed that telomerase re-expression linked with the carrying mutations at two hotspots in the *TERT* promoter. Alterations found in these regions are the most frequent recurrent somatic mutations in LGDNs (6 %), HGDNs (20%) and HCC (60 %), respectively [121, 132]. Thus, *TERT* promoter activation is required at an early step of transformation to bypass the replicative senescence of cirrhotic hepatocytes. In contrast to the early stage, diverse genomic alterations also involve the progression of disease during the late stage of liver carcinogenesis [133]. In HCC progression, telomerase overexpression in 90 % of HCC facilitate the maintenance of telomeres and contributes to the evasion of hepatocyte senescence. Overexpression of telomerase often occurs due to *TERT* promoter mutations in almost 60 % of cases. However, *TERT* gene amplification also reported in less amount of the patient who is around 5 % [110, 121, 132].

Mutations often located at the nucleotide position -124 and -146 upstream of the ATG in *TERT* promoter. Presence of these mutations led to a new binding site which can be recognized by transcription factors and further induce *TERT* expression. Beside telomerase activation, WNT- β -catenin pathway is also frequently activated in HCC. Mutations in β -catenin (*CTNNB1*) reported in the range of 11-37 % of the total HCC cases, particularly among the patients who exhibited no HBV infections and carried well-differentiated tumors [134, 135].

Besides, deletions relatively high ratio of inactivating mutations occur in axin 1 (*AXIN1*) almost around 10 % of HCCs, yet mutations rarely seen in adenomatous polyposis coli (*APC*) and zinc and ring finger 3 (*ZNRF3*) both less than 3 % of HCC cases. All these mutations often led to an activation of WNT- β -catenin pathway [121, 132]. In addition to telomerase and WNT- β -catenin activation, inactivation of p53 and alterations in the cell cycle drives the progression of HCC.

HBV and HCV infections are also associated with the early changes for the malignant transformation [136]. Specific genomic alterations identified mostly upon HBV infection. Integration of HBV into hepatocytes genome induces the carcinogenic by activating major driver genes [137, 138]. The most frequent insertions of HBV DNA in hepatocytes occur within the *TERT* promoter and activate telomerase and various other oncogenes including cyclin E1 (*CCNE1*), lysine (K)-specific methyltransferase 2B (*KMT2B* or also called *MLL4*) and SUMO1/sentrin-specific peptidase 5 (*SEN5*) [21]. Integration of HBV in *TERT* has identified more than 20 % HBV-related HCC patients where gene interrupted [139]. Surprisingly most of these insertions occur in the promoter region of the *TERT* which support its re-activation [140].

p53 and retinoblastoma (Rb) pathways are strongly associated with HCC. Both loss help to bypass hepatic cells from cell cycle arrest and quiescence, which provoke fibrogenesis, and activation of stellate and hepatic progenitor cells upon liver damage. Loss of Rb and lack of functional p53 associated with many human tumors [141]. p53, called as the guardian of the genome, is one of the well-known tumor suppressor gene and almost half of the human tumors exert deregulations in the p53 pathway. *TP53* gene is mostly mutated and inactivated in tumors independent to their tissue origin and etiology [142]. There are several mutational hot spots have been documented, especially in the DNA binding domain of the p53 [143]. Mutation in *TP53* is almost around 30 % in HCC with a hyper variability between different geographical areas [144]. For instance, AFB1 exposure caused *TP53* mutation on codon 249, leading to a substitution of arginine to serine (R249S) highly correlated at the particular region of the world [145]. In addition to AFB1 effect on *TP53*, several studies also suggested that p53 could inhibit by viral proteins [146, 147]. Besides the inactivation, p53 protein level or function also altered the rest of the tumors. Under the basal condition, the cellular p53 level is too low and upregulated upon stress, including DNA damage and chemotherapeutic exposure.

In HCC, several studies revealed that both p53 mutation and inactivation play a key role in disease progression. In line with this, *TP53* mutations and deregulation of p53 expression also documented to be correlated with tumor stage. Thus, the role of p53 suggesting is a late event in hepatocarcinogenesis [148] Moreover, an elevated level of ROS also investigated in the p53 pathway, and studies showed that increased level of ROS commonly associated with *TP53* mutations in HCCs [149]. Of note, ROS associated with hepatocarcinogenesis, which increased the progression of cirrhosis and further HCC.

In the p53 pathway, Mdm2 is one of the well-known partners where binds and control its degradation in cells. Promoter SNP309 found in Mdm2 gene, which is also reported to be a risk factor of HCC [150]. In addition, promoter methylation of p14, cell cycle inhibitor and senescent associated protein, identified in 19 % of HBV-related HCCs and 39 % of alcohol-related HCCs [151]. As a downstream gene of p53, p21 transcription often altered in HCC patients [152]. Viral proteins such as HCV core protein affect the expression of p21 in a p53-dependent manner [153, 154]. p27 is another cell cycle inhibitor whose expression level decreased via different mechanisms in [155]. Absence of p27 displayed hyperplasia-like phenotype, which the number of hepatocytes increased in the liver of transgenic mice [156]. However, hyperplasia does not follow HCC in this mice model under healthy liver conditions.

pRb is another tumor suppressor which control cell cycle progression through repressing the transcription factors [157]. Mutations in *RBI* involved 3-8 % of HCC cases. In line with this, cyclin-dependent kinase inhibitor 2A (*CDKN2A*) deletions comparably higher in HCC than *RBI*, which is around 12 %. All these alterations strongly associated with the poor prognosis and aggressive phenotype of the disease [121, 158]. In HBV-related HCCs, pRb expression was altered and almost not detected in the quarter of the analyzed patients. Additionally, gankyrin is responsible for proteasomal degradation of pRb, and its overexpression reported in HCCs. Cyclin D1 (*CCND1*) and *FGF19* are bona fide oncogenes in 11q13 where this specific genomic region highly amplified in 5-10 % HCCs [159].

Deregulation in the expression of CDK inhibitors p16, p21, and p27 often related to the cell cycle-assisted control of hepatocarcinogenesis, both altered as 90 % of HCCs [160]. In general, p16 inactivated during the early stage of HCC. Epigenetic silencing of the p16 gene by promoter methylation is frequently associated with its inactivation and HCC [144].

Alterations in epigenetic regulators and chromatin remodeling complex components mainly addressed in advanced-HCC. For instance, HBx alters the expression of DNA methyltransferases (*DNMT1* and *DNMT3A*) to trigger hypermethylation of p16 in HCC [141, 161]. BRG1-or HRBM associated factors (*BAFs*), and polybromo-associated BAF (*PBAF*), AT-rich interaction domain 1A (*ARID1A*) and *ARID2* are one of the most frequent mutated genes in this context. Histone methylation writer family (KMT2 or also known as MLL) family member genes also mutated around 2-4 % of total HCC cases. Of note, HBV integration within *KMT2B* reported in 10 % of the HCC cases [162].

RAS-RAF-MAPK (MAP kinase) and the phosphoinositide 3-kinase (PI3K)-AKT-mTOR pathways also activated in HCC. Amplification of fibroblast growth factor 3 (*FGF3*), *FGF4* and *FGF19*, inactivating mutation in tuberous sclerosis 1 (*TSC1*) or *TSC2* and phosphatase and tensin homolog (*PTEN*) reported around 5, 3-8 and 1-3 % of the tumors, respectively. In contrast, mutations that activate RAS proteins themselves (*KRAS*, *HRAS*, *NRAS* or *BRAF*) rarely identified in HCC [120].

Oxidative stress-associated pathways constitutively activated in HCC due to an activating mutation in nuclear factor erythroid 2-related factor 2 (*NFE2L2*) or inactivating mutation in Kelch-like ECH-associated protein 1 (*KEAP1*) with a frequency of 5–15 % of total HCC cases. Upon liver damage, transforming growth factor- β (TGF- β) produced by hepatic stellate cells and blocking the growth of hepatocytes by inhibiting DNA synthesis and inducing apoptosis. In hepatocytes, TGF- β induces the expression of death-associated protein kinase (DAPK) and control cell death[163]. Moreover, recent reports suggest that TGF- β also facilitates to fibrinogenesis, which further support cirrhosis. β -catenin is also associated with epithelial-mesenchymal transition (EMT) in HCC. In the absence of WNT ligand, it binds to the E-cadherin at the adherent junctions.

Sequestration of β -catenin at the junctions alter cytoskeleton and facilitate the cell polarity further involves cancer cell migration and invasion. Besides, nuclear accumulation of β -catenin repressed the expression of E-cadherin [164].

1.2 Autophagy

1.2.1 Mechanisms of autophagy

Autophagy is orchestrating by several consecutive steps, which required the activation of several individual proteins called autophagy-related proteins (ATG) (**Figure 1.6**). Several protein complexes found to be responsible for the activation of autophagy upon various stimuli [2].

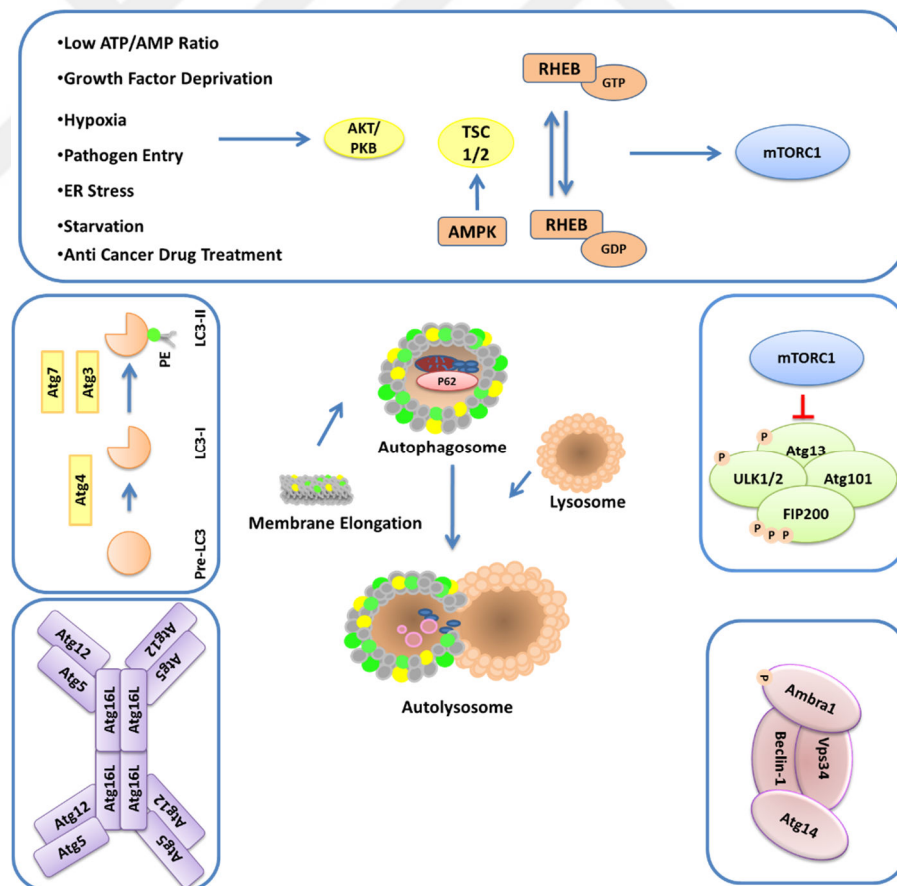


Figure 1. 6 Autophagic regulation by several protein complexes.[165]

Protein complexes containing the serine/threonine kinase mTOR are among the central regulators of autophagy under stress conditions, including amino acid deprivation [166]. mTOR found in two distinct complexes: mTORC1 and mTORC2. Both compounds were associated with the regulation of autophagy[167, 168]. The mTORC1 complex composed of mTOR kinase, mLST8, DEPTOR, Tti/Tel2, Raptor, and PRAS40 proteins. mTORC2 also contains the mTOR kinase, mLST8, Tti/Tel2, and DEPTOR, and additional components Rictor and mSin1 [169]. Stress leads to the activation of autophagy following inhibition of mTOR complexes. Under basal conditions, ATG13 and ULK1 are phosphorylated by mTORC1, leading to their suppression [170]. Upon nutrient deprivation, mTORC1 inhibited, and ATG13 and ULK1 dephosphorylated and activated. Autophosphorylation of ULK1 further activates the kinase and results in the phosphorylation of autophagy proteins, including ATG13 and FIP200 [170, 171].

mTORC1, as a nutrient sensor, was found on lysosomes. Lysosomal recruitment of the complex-mediated by the RAG family of GTPases in response to amino acid availability. Phosphorylation of TFE/MITF family of transcription factors by lysosome-associated mTORC1 results in their sequestration in the cytosol and inactivation. An increase in the amino acid release from the lysosomes leads to the detachment and inactivation of mTORC1, and result in the dephosphorylation and nuclear translocation of TFE/MITF transcription factors. Transcriptional programs that activated in this context promote lysosomal biogenesis and autophagy [172, 173].

In addition to amino acid deprivation, glucose starvation and declining cellular energy levels are also able to induce autophagy. AMPK, intracellular AMP/ATP ratios sensory serine/threonine kinase play a crucial role in this context. A decline in ATP levels results in the accumulation of AMP that, in turn, binds and allosterically activates AMPK [174]. AMP binding enhance the phosphorylation by upstream kinases CaMKKb and LKB1 (STK11), further activating the kinase [114, 175]. AMPK blocks mTORC1 activity through phosphorylation-dependent activation of tuberous sclerosis 2 (TSC2) complex and inhibition of Raptor [176]. Besides, AMPK may directly activate autophagy through phosphorylation and activation of ULK1 [177].

The type-III PI3 kinase, VPS34 controls the nucleation stage of autophagy. A complex containing VPS34, Beclin-1, Atg14, Vps15, and AMBRA1 leads to the phosphorylation of membrane-associated phosphoinositol lipids (PI), resulting in the accumulation of phosphoinositol-3-phosphates (PI3Ps) on the cytoplasmic surface of intracellular membranes. Endoplasmic reticulum (ER), Golgi and the plasma membrane counted among membrane-bound compartments from which autophagosomes may originate [178-180]. PI3P on the membranes recruits lipid-binding proteins that mark the nucleation sites, such as PIP3P DFCP1 and WIPI proteins.

Two ubiquitylation-like conjugation systems identified to involve in the elongation of isolation membranes: The ATG12-5-16 system and The ATG8/LC3-lipid conjugation system. In the first system, upon ATG12 activation by E1-like ATG7, ATG5 is conjugated to ATG12 with the help of the E2-like protein ATG10 [178]. Then, ATG12-5 conjugates interact with ATG16L, and the forming complex serves as an E3-like function in the second conjugation system [181, 182]. Here, reactions involving E1-like enzyme ATG7 and E2-like enzyme ATG3 result in the activation of ATG8 proteins such as LC3, GATE-16, and GABARAP1/2. ATG12-5-16 conjugate serves as an E3 ligase and facilitates conjugation of ATG8 proteins to lipid molecules such as phosphatidylethanolamine (PE) [178]. Conjugation of ATG8 proteins to PE promotes isolation membrane expansion and autophagic vesicle completion [183]. For lipid conjugation reactions to occur, ATG8/LC3 proteins should be primed by ATG4 proteins through C-terminal cleavage [184, 185].

Autophagosome lysosome fusion and autophagosome maturation require several complexes such as SNARE proteins, integral lysosomal proteins (e.g., LAMP-2) and RAB proteins (e.g., RAB5 and RAB7). SNARE proteins include syntaxin 17 (STX17), SNAP29 and VAMP8, and the homotypic fusion and protein sorting (HOPS) complex is also involved in the process. Docking and fusion controlled by RAB5, RAB7, and LAMP2 proteins [186]. In addition to non-selective and constitutive autophagic activity, selective forms of autophagy also described. A number of autophagy receptors have been discovered, including SQSTM1/p62 [187], NBR1 [188], NDP52 (also known as a CALCOCO2) [189], OPTN [190] and NIX (also known as BNIP3L) [191]. The receptors generally form a bridge between ATG8/LC3 proteins and cargo molecules and promote their preferential engulfment.

1.2.2 Autophagy in HCC

The role of autophagy is quite involved in the cancer context, and its role in HCC is fundamental [165]. There is experimental evidence that in early phases of cancer formation, autophagy functions as an anti-cancer pathway, preventing malignant transformation of healthy cells to cancer cells. On the other hand, autophagy involved in various stages of cancer progression, including metastasis. Notably, the survival of fast-growing tumors correlated with their autophagic activity. An extensive collection of articles implicating autophagy in drug resistance context as well.

Protein metabolism is one of the primary function of the liver, including fat and carbohydrate metabolism. Autophagic regulation and cellular degradation processes are as necessary as an early genetic alteration in hepatocytes, including telomerase re-activation, oxidative stress, and WNT- β -catenin pathway activation. Several different stages of HCC progression manifests in autophagy null mice according to the absence of the gene itself. *Atg6/BECN1* (Beclin 1) is an essential gene in the initiation of the autophagy pathway. Deletion of the *BECN1* observed in 40-75 % of human cancers [192]. Increased tumorigenesis reported upon heterozygous deletion of *atg6/beclin1* in multiple mice tissues, including liver [192, 193]. Moreover, *becn1* null mice exhibit accelerated HBV-related HCCs [192]. Besides *becn1* removal of other autophagy genes, such as *atg5* and *atg7*, led to a formation of benign liver adenomas in mice models [194]. However, liver-specific deletion of *atg7* resulted in hepatomegaly and hepatic failure in mice, not to developing a liver malignancy. Therefore, homeostasis of healthy liver suggested to controlled by autophagic activity perturbation of which might be the cause of HCC.

Surprisingly, additional deletion of *p62* in a liver-specific *atg7* deficient mice, alleviated tumor burden, indicating that an essential role of autophagy in this context is to eliminate cellular protein aggregates in a p62-dependent manner [195]. In as much as many other crucial genes involved in HCC, some of the autophagy-related genes also increase the susceptibility of HCC development upon liver damage. Deletion of autophagy-related genes *Uvrags* enhanced sensitivity to HCC development in mice [196, 197].

Autophagy takes place in the clearance of damaged mitochondria, elimination of abnormal and mutant proteins and protein aggregates, and specific elimination of proliferation-related proteins. Thereby, autophagy also suggested as cancer-preventing mechanisms in contrast to its tumor-promoting phenotype [3, 198]. For instance, a specific type of autophagy called ‘mitophagy’ facilitate the clearance of damaged mitochondria, the primary source of endogenous ROS in cells. Accumulation of ROS, one of the significant driver in hepatocyte damage and subsequent cirrhosis, occurs upon perturbances in autophagic activity and increase DNA damage and genomic instability. Besides, activation of the NRF2, which is one of the critical transcription factors in antioxidant defense, was found to be regulated by autophagy [199]. Under normal conditions, Keap1, an adaptor protein of Cullin-3 ubiquitin ligase, allows ubiquitination and degradation of NRF2. Upon ROS accumulation results in the oxidation of Keap1 and its dissociation from NRF2, leading to its stabilization and nuclear migration. Moreover, competitive binding of the autophagy receptor p62 to Keap1 followed by their selective autophagic degradation resulted in the activation of NRF2, which eventually trigger an antioxidant transcriptional pathway. In line with this, the accumulation of p62 was found to drive liver cancer formation in several mice models [194, 195, 200]. Of note, activating mutations in NFE2L2 and inactivating mutations within KEAP1 found almost around 5-15 % of HCC cases [121]. All these emphasize the protective role of autophagy in hepatocarcinogenesis (**Figure 1.7**).

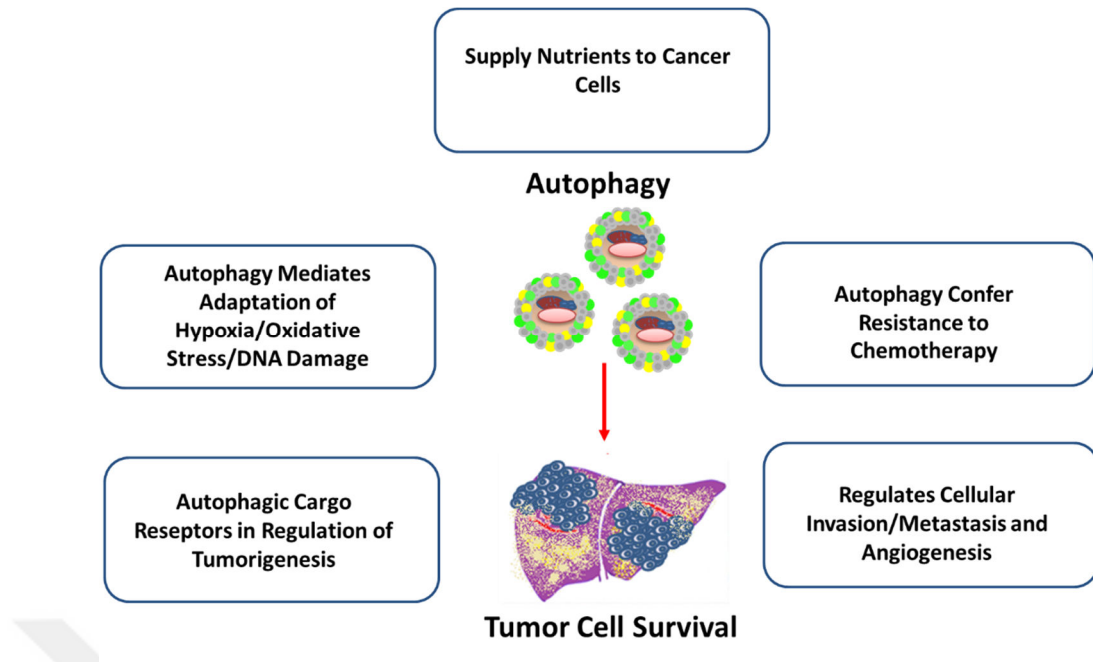


Figure 1. 7 Tumor promoter roles of autophagy in HCC [165].

In contrast, rather than its protective role, autophagy also crucial in the progression of established malignancies. Basal autophagy was elevated in hypoxic regions of some solid tumor types and found to be an essential role for tumor cell survival in experimental models [201].

In the case of unlimited tumor neovascularization, especially in fast-growing tumors, regions that have limited access to nutrients and oxygen supply [202]. Thus, cancer cells in these regions might be more dependent on autophagy than healthy growing cells. Autophagy is also associated with the survival of cancer cells in circulation and contribute to metastasis and chemotherapy resistance (**Figure 1.8**).

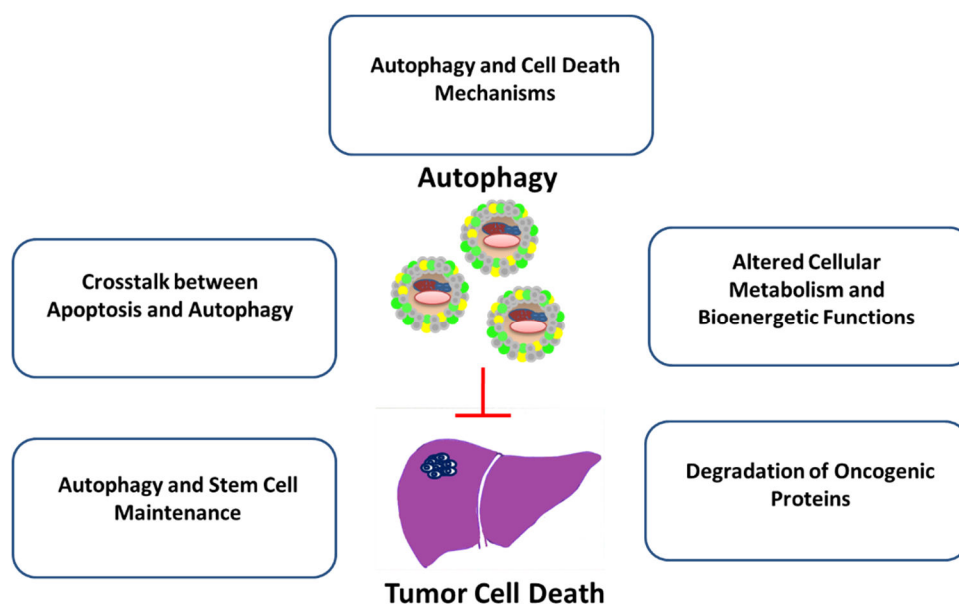


Figure 1. 8 Tumor promoter roles of autophagy in HCC. [165].

1.2.2.1 Autophagy in HCC therapy

HCC is one of the fatal and most frequent cancer worldwide. A predisposition of HCC development in patients, mainly underlying chronic liver disease and cirrhosis. Therefore, understanding the molecular mechanisms hepatocarcinogenesis and the contribution of autophagy to these mechanisms are among the essential challenges of modern medicine.

Sorafenib is an FDA-approved tyrosine kinase inhibitor (TKI) which widely used in the treatment of advanced-stage HCC [203]. However, its effect of OS is limited [204]. In the mode of action, Sorafenib induces cell death and caused ER stress in HCC cells [204]. Moreover, sorafenib demonstrated to inhibit mTOR and led to an accumulation of autophagosomes [204]. Concomitantly, sorafenib exposure upon autophagy attenuation using CQ or following genetic suppression by a specific siRNA (small interfering RNA) exhibit synergistic effects on tumor growth suppression [205].

Besides, its combination with proteasome inhibitors significantly increased HCC cell death compared to sorafenib alone, which also highlight the importance of both degradation systems in HCC survival [206].

Another TKI, nilotinib also stimulated autophagy in HCC cells through AMPK phosphorylation and regulation of PP2A [104, 207]. Bevacizumab is another targeted drug against VEGF, also found to increase autophagy. In as much as sorafenib, its exposure following autophagy suppression increase HCC cell death.

Conventional chemotherapeutic agents were also shown to activate autophagy in several cancer types. For example, oxaliplatin is a Platinum-based chemotherapy agent which is widely using in the treatment of HCC led to autophagic activation in both HCC cells and xenografts [208]. Inhibition of autophagy under these conditions increased the cytotoxicity of oxaliplatin [208], another study, cisplatin, and 5-FU induced the formation of autophagosomes HCC cell lines, and attenuation of autophagy enhanced the cisplatin and 5-FU-induced cell death under both in vitro and in vivo conditions

Inactivating mutation within the gene of *TSC1* or *TSC2* and *PTEN* and upregulation of mTOR signaling identified in a significant proportion of HCCs. RAD001 and BEZ235 characterized as PI3K/mTOR inhibitor drugs. Combination of both achieves to suppress HCC growth both in vitro cell culture and in vivo in mice experiments [209]. Another molecule, the Akt inhibitor MK-2206 was also found to trigger cell death, and suppression of autophagy alongside inhibitor, further enhanced the cell death in HCC cells [210]. In contrast to protective autophagy, high-LET irradiation showed to kill HCC cell in an autophagy-dependent manner both in vitro and in vivo conditions [211]. Moreover, RAD001 combination with high-LET radiation elevated the cell death in HCC cells [212].

1.2.2.2 Autophagy and HCC through miRNAs

In addition to protein-coding genes, the genome of various organisms, carry the genes of multiple non-coding RNAs, including snoRNAs, piRNAs, lncRNAs, circRNAs, etc. Among them, miRNAs are a class of small RNAs that are found either in intergenic regions of genomes or in the intronic regions of protein-coding genes [213-215].

Silencing is a result of a blockage of translation, mRNA sequestration in P-bodies or mRNA degradation. Degree of complementarity between miRNA and mRNA target sequences (miRNA response elements, MREs) is critical for the miRNA action.

MiRNAs are usually able to bind to more than one mRNA species and control the expression of several of different transcripts at once [216, 217]. By this way, to modulate important biological events, miRNAs can control several targets on the same pathway at once as well as several parallel independent pathways.

The autophagic activity has to keep under strict control in cells. Excessive or defective autophagic activity is not compatible with cellular and organismal homeostasis, and aberrant autophagy was generally associated with a wide range of pathologies such as cancer and neurodegenerative diseases.

MiRNA levels are sensitive to a variety of conditions that lead to cellular and/or organismal stress. Up or down-regulation of miRNAs above or below certain thresholds in response to stress signals may result in the activation of cellular stress response pathways, including autophagy. On the other hand, autophagy-inducing stress conditions (e.g., starvation, energy crisis, growth factor deprivation, etc.) associated with modulation in the levels of several miRNAs [218]. Conversely, miRNA machinery components and even individual miRNAs were selectively sequestered and degraded by the autophagy-lysosome system. By this way, through a check and balance system, autophagy might control its own regulation.

Many miRNAs were shown to regulate the induction stage of autophagy. The gene of mTOR, a central regulator of autophagy, was described as the target of several miRNAs in the autophagy context, including *MIR100*, *MIR96*, *MIR101*, *MIR199A-3p* [219-222]. Almost all core autophagy pathway elements were subject to miRNA-dependent fine-tuning. For instance, in melanoma cells, targeting ULK1 by *MIR290/295* resulted in the suppression of autophagic activity in response to glucose starvation [223]. Regulation of ATG14 by *MIR195* suppressed nerve damage-induced autophagy in the context of nerve inflammation and neuropathic pain [224]. Moreover, *MIR214* was found to target ATG12 and regulate radiation-induced autophagic flux in colorectal cancer cells [225]. In breast, liver cancer and leukemia cells, overexpression of *MIR181A* resulted in a significant decrease in starvation and rapamycin-induced autophagy through targeting of ATG5 [226]. Two distinct studies from our group demonstrated regulation of ATG4C by the *MIR376* family. *MIR376A/B* regulated of mTOR-dependent autophagy in breast and hepatocellular carcinoma cancer cells through direct targeting of ATG4C and BECN1 [227, 228].

ATG8/LC3 genes also regulated by miRNAs. For instance, *MIR204*, a miRNA that was governed by the tumor suppressor VHL in a renal carcinoma context, directly controlled the levels of LC3 and determined the levels of basal autophagy [229]. In another study, a SILAC proteomics approach led to the identification of SQSTM1 as a target of *MIR17/20/93/106* in the myeloid progenitor cell line. AML-related upregulation of *MIR106* correlated with a decreased level of SQSTM1 and higher mortality rates in patients [230]. Another miRNA, *MIR4459*, inhibited of the expression of the LARP1 transcription factor that is involved in SQSTM1 expression in vascular endothelial cells [231].

MiRNAs were also reported to regulate the maturation step of autophagy. Frankel, et al. revealed the role of *MIR101* in the regulation of autophagy through multiple targets, including RAB5A [232]. RAB1B1 described as another miRNA target. Similarly, RAB27A levels depended on *MIR205*. Additionally, the same miRNA also controlled lysosome integral protein LAMP3 as well. Another lysosome protein, LAMP2, was reported to be a target of more than one miRNAs, namely *MIR207*, *MIR352*, *MIR487-5p* [233-235].

The growing number of miRNAs deregulated in several disease-related conditions. Dysregulation of autophagy-related miRNAs was also associated with several diseases, including cancer, neurodegeneration, cardiovascular disease, and infections [218]. MiRNAs in HCC also introduced as disease markers and even gene therapy tools. Drug treatments also resulted in changes in the cellular amounts of specific miRNAs or miRNAs facilitate the overcome the drug-resistance in HCC (**Table 1**).

Table 1. Autophagy-regulating miRNAs in HCC

miRNA	Cell Line	Autophagy-Related Target	Autophagy-inducing stimuli	References
MIR100	HepG2	MTOR and IGF-1R	N.D.	Ge et al., 2014
MIR376B	MCF7, Huh-7	ATG4C and BECN1	Starvation, Rapamycin	Korkmaz et al., 2012
MIR376A	MCF7, Huh-7	ATG4C and BECN1	Starvation	Korkmaz et al., 2013
MIR181A	MCF7, Huh-7	ATG5	Starvation, Rapamycin	Tekirdag et al., 2013
MIR101	HepG2	RAB5A, STMN1, ATG4D	Cisplatin	Xu et al., 2013
MIR199A-5p	HepG2, Huh-7	ATG7	Cisplatin	Xu et al., 2012
MIR141	HepG2	SIRT1	HBV infection	Yang et al., 2017
MIR181-5p	HST-T6	STAT3, BCL2	N.D.	Qu et al., 2017
MIR30A	HSC-T6, LX-2	BECN1	TGF- β 1	Chen et al., 2017
MIR30A	HCCLM3 and Huh7	BECN1, ATG5	N.D.	Fu et al., 2018
MIR185	HepG2	AKT1 RHEB RICTOR	N.D.	Zhou et al., 2017
MIR26A/B	HepG2	ULK1	Doxorubicin	Jin et al., 2017
MIR7	Huh-7, HepG2	MTOR	N.D.	Wang et al., 2017
MIR96-5p	LX-2	ATG7	Rapamycin	Yu et al., 2018

For example, cisplatin treatment found to reduce the levels of drug resistance-associated *MIR199A-5p* in HCC patients and *MIR199A-5p* attenuates cisplatin-induced autophagy through targeting of ATG7 [236]. In addition, *MIR375* is also another miRNA targeting ATG7 was downregulated in HCCs and decreased the viability of HCC cells under hypoxic conditions [236, 237]. *MIR224* is one of the most studied miRNAs in HCC. It targets Smad4. Strikingly, high *MIR224* levels were also associated with lower Atg5. *MIR224* level significantly associated with HBV infection and poor overall survival in HCC patients [238]. Interestingly, autophagy itself limited *MIR224* levels through direct degradation of the miRNA, hence resulting in liver tumor suppression [238].

MIR101 also characterized as an autophagy inhibitory miRNA, and it plays a role in several chemotherapeutic drug-induced cell deaths in HCC [239, 240]. More than that, there are several other miRNAs have been addressed in the sorafenib resistance context in HCC.

For instance, among these miRNAs, *MIR21* was found to suppress autophagy via PTEN/Akt axis and lead to sorafenib resistance [241]. Besides, Sorafenib-induced miRNA deregulations also crucial for the determination of prognosis and follow-up purposes. *MIR423-5p* described as a positive regulator of autophagy in HCC cells and also associated with sorafenib response following treatment [242]; Besides, *MIR30A* levels were affected during HBV infection [243]. Overexpression of *MIR30A* could overcome HBx-induced *BECN1* upregulation and inhibited autophagosome formation in HepG2 liver cancer cells. HBV-related autophagy could be inhibited by *MIR30A*, suggesting that the miRNA levels may be a critical determinant of responses to HBV-related HCC [243].

MIR100 introduced as an autophagy-related miRNA in HCC. Stable *MIR100* expression attenuates the tumor growth in xenograft compared to controls, and *MIR100* expression levels were found downregulated in analyzed human HCC tissue samples. Hence, induction of autophagy, as well as apoptosis, was observed in cells with high *MIR100* levels, revealing the role of the miRNA in cancer suppression by direct targeting of mTOR and IGFR1 [219]. More than that, ULK1 has also been considered as a target of another miRNA in HCC. *MIR141*, inhibited autophagy by targeting Sirt1, conferring inhibitory effect of HBV replication. *In vitro* studies showed that *MIR141* mimic inhibited the autophagic response, HBV load, and the expression of Sirt1 in hepatocytes. Besides, introduction to *MIR141* inhibitor in HCC cells enhanced autophagy [244]. *MIR185*, is another autophagy inducer miRNA through targeting both RHEB, RICTOR, and AKT1 by directly interacting their 3' UTRs. In HCC cells, *MIR185* has been associated with cell proliferation while overexpression suppresses HepG2 cell growth and induce apoptosis [245].

According to our knowledge, hepatocarcinogenesis involved in many cells in the liver besides hepatocytes. For instance, in cirrhosis hepatic stellate activation and fibrinogenesis predominantly lead the disease progression. *MIR96-5p* is one of the miRNA studied in this context. *MIR96-5p* level found to increase in fibrotic liver tissues. Overexpression caused a decrease of ECM-related proteins, including α -SMA and Collagen 1 in parallel to autophagy in LX-2 cells. In contrast, re-introduction of *MIR96-5p* target gene ATG7 into LX-cells rescue the phenotype [246]. Upon damage, hepatic stellate cells (HSCs) activated and support scarring procedure through secreting collagen. *MIR181-5p* overexpressed in adipose-derived mesenchymal stem cells (ADSCs).

These cells used to occur exosomes which reside *MIR181-5p*. Then, ADSCs-derived *MIR181-5p* containing exosomes introduced to the mouse hepatic stellate (HSC-T6) cells or a CCl₄-induced liver fibrosis murine model to analyze the effect of tumor microenvironment via exosomal miRNA transfer. Exosomal *MIR181-5p* targets STAT3/Bcl-2/Beclin 1 follow the induction of autophagy and reduction of TGF- β 1-induced liver fibrosis in HSC-T6 cells [247]. In another study, *MIR30A* overexpressed in mouse HSC-T6 and human LX-2 HSCs. *MIR30A* overexpression in HSCs provoked downregulated ECM including α -SMA, TIMP-1, and Collagen 1 expression and further suppressed cell viability. HSCs-derived exosomes then injected to the mice, and exosomal *MIR30A* achieved to decreased TGF- β 1-induced liver fibrosis in mice [248].

1.2.2.3 *MIR376 family and HCC*

MIR376 gene family located on 14q32 and consist of seven different miRNAs named as; *MIR376A1-A2*, *MIR376B*, *MIR376C*, *MIR654*, *MIRB1*, and *MIRB2* [249]. All these family members shared almost the same seed sequence either fully or partially with a few base changes. However, *MIR654* has completely different seed sequence in comparison with the other family members. Thus all may act on the same gene and exert similar cellular functions.

All the members transcribed as a single long primary transcripts [250, 251]. In our laboratory, we identified 3 of these miRNAs as autophagy-regulating miRNAs [227, 228]. In contrast, individual miRNAs from this family act differently with a kinetic manner under different stress conditions [228]. *MIR376B* family have studied in several cancers [252]. In particular, *MIR376A* shown to downregulated in early phases of liver regeneration [253]. In line with this, the expression of *MIR376A* targets p85a (*PI3K3R1*) and suppressed cell proliferation and induced apoptosis in HCC. The same group also revealed the epigenetic regulation of *MIR376A*. Histone deacetylases (HDAC) inhibitors increased the expression of *MIR376A* [254]. *MIR376A* targets HDAC9 and knockdown of the HDAC9 led to the upregulation of *MIR376* family members [255].

In our hand, both miRNAs found to decreased autophagy by targeting BECN1 and ATG4C in breast and HCC cell lines. In our previous studies, we also identified that both miRNA *MIR376A* and *MIR376B* have different kinetics upon various stimuli, including starvation and mTOR inhibitor rapamycin. Re-introduction to targets in cells rescue the miRNA dependent phenotype, which suggests the direct involvement of the miRNA [228].



2 MATERIALS AND METHODS

2.1.1 Plasmid constructs

The pMSCV-miR plasmids were containing 443 different human miRNA minigene sequences mimicking primiRNAs constructed as described previously. hTR (human telomerase RNA) was cloned into the same plasmid and used as control [228].

2.2 Cell culture

Huh7 human hepatocellular carcinoma cells were cultured in DMEM (Sigma, 05671) supplemented with 10% (v/v) fetal bovine serum (FBS; Biochrom KG, S0115) and antibiotics (Penicillin/Streptomycin; Biological Industries, 03-031-1B) in a 5% CO₂-humidified incubator at 37°C. In order to induce autophagy, starvation in Earle's Balanced Salt solution (EBSS; Biological Industries, BI02-010-1A) used.

2.3 Generation of Huh7 stable cells

Generation of Huh7 stable cell lines, we first started with Blasticidine S Hydrochloride (Sigma, 15205) kill curve analysis. 1-25 µg/ml Blasticidine was administered for liver cancer cells in 24-well plate. The cells were checked regularly under microscope for 7 days and 10 µg/ml dose killed all cells at 7 days for both cell lines.

In order to generate stable cell line, cells were transfected with AMAXA nucleofector (Lonza Nucleofector II AAD-1001S) with program E-014 and the Nucleofector kit V (Lonza, VCA-1003) according to the manufacturer's instructions with 5 µg of HTR or MIR376B plasmid only. 72 hours after final transfection concentration: 10 µg/ml blasticidine given in DMEM+/+/+. Selection with blasticidine continued for 4 weeks as polyclonal population will arise and limiting dilution will be carried out for 0.5-1 cell/well of 96well plate to obtain monoclones.

5 - 96 well plates filled for all clones of HTR, MIR376B stable cells. Monoclones that grew in 96 wells were trypsinized and taken to 24- to 12- to 6-well plates and frozen as cell stocks in 10% DMSO.

2.4 Protein extraction, Immunoblot analysis and antibodies.

Protein extraction was performed with RIPA buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate) supplemented with complete protease inhibitor cocktail (Roche, 04-693-131-001) and 1mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, P7626). For phosphorylated proteins, protein extraction was performed with RIPA buffer supplemented with complete protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, P7626) and 100 nM okadaic acid, 1 μ M cyclosporine A, 1 mM NaF, 50 mM β -glycerophosphate. Cell extracts (30-50 μ g) were separated in 15-10% SDS-polyacrylamide gels and transferred to PVDF membranes (Roche, 03010040001). Following blockage in 5% non-fat milk in PBST (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl and 0.05% Tween 20, pH 7.4) for 1 hr at RT, membranes were incubated in 3% BSA-PBST solutions containing primary antibodies (ab): anti-LC3B ab (Sigma-Aldrich, L7543, dilution 1:2000 or Novus, 2331, dilution 1:1000), anti-SQSTM1/p62 ab (BD Transduct. Lab, 610832, 1:1000 or Abnova, H00008878, 1:4500) anti-ACTIN/ACTB ab (Sigma-Aldrich, A5441, dilution 1:7500), anti- ATG4C (Sigma, A9482,1:2000), anti- BECN1(Santa Cruz, SC11427, 1:1000). Then, secondary mouse or rabbit antibodies coupled to horse-radish peroxidase (anti-mouse: Jackson ImmunoResearch Laboratories, 115035003; anti-rabbit: Jackson ImmunoResearch laboratories, 111035144, dilutions 1:10.000) were applied in 5% milk/PBST for 1 hour at RT and protein bands were revealed with chemiluminescence. Band intensities were quantified using ImageJ software.

2.5 GFP-LC3 dot analysis

To detect autophagy activation by GFP-LC3 dot analysis, GFP-LC3 plasmid transfected to the cells by PEI. After 48 h cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, 15,812-7), mounted and inspected under 60× magnification using a BX60 fluorescence microscope (Olympus, BX60, Japan). Basal autophagy threshold determined as 15 GFP-LC3 dots per control-treated GFP-LC3 MEF cells. Dots in at least 150 cells per experimental point were counted and analyzed. Graphs were plotted as the percentage of GFP-LC3 dot positive cells over total cell numbers.

2.6 RNA isolation and RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Sigma-Aldrich, T9424) according to the manufacturer's instructions. cDNA was reverse transcribed from total RNA (DNase treated) using M-MuLV reverse transcriptase (Fermentas, EP0351) or RevertAid enzyme (ThermoScientific, EP0441) and random hexamers (Invitrogen, 48190-011).

2.7 Real-time RT-PCR for BECN1 and ATG4C mRNA quantification

SYBR Green Quantitative RT-PCR kit (Roche, 04-913-914-001) and an iCycler iQ thermal cycler (BioRad) or Roche Light Cyclers 480 used for single-step qRT-PCR reactions. To activate the SYBR green, an initial cycle of 95°C, 10 min performed followed by, PCR reactions: 40 cycles of 95°C for 15 sec and 60°C for 1 min. Then a thermal denaturation protocol was used to generate the dissociation curves for the verification of amplification specificity (a single cycle of 95°C for 60 sec, 55°C for 60 sec and 80 cycles of 55°C for 10 sec). Changes in mRNA levels were quantified using the $2^{-\Delta\Delta CT}$ method using *GAPDH* (Gyceraldehyde-3- phosphate dehydrogenase) mRNA as control.

Primers used during the study were:

GAPDH primers 5'-AGCCACATCGCTCAGACAC-3';

5'-GCCCAATACGACCAAATCC-3';

BECN1 primers 5'-AGGTTGAGAAAGGCGAGACA-3';

5'-GCTTTTGTCCACTGCTCCTC-3' ;

ATG4C primers 5'-GCATAAAGGATTTCCCTCTTGA-3' ;

5'-GCTGGGATCCATTTTTCG-3', Reactions were performed in duplicates and the number of independent experiments (n) was marked.

2.8 TaqMan RT-PCR for endogenous MIR376A, MIR376B quantification

TaqMan qRT-PCR reactions were performed using FastStart Universal Probe Master kit (Roche, 04913957001) and an iCycler iQ thermal cycler (BioRad) or Roche Light Cycler 480 according to the protocols described previously[228]. Primers and the probe used during the study were:

Universal Reverse primer, 5'-GTGCAGGGTCCGAGGT-3'.

Stem loop primer for MIR376B:

5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACGTGG
ATTTTCCTCTATGAT-3';

Forward primer for MIR376B: 5'-ATTAATCATAGAGGAAATCCACG-3';
Reverse primer for MIR376B: 5'-GTGCAGGGTCCGAGGT-3'; Probe for MIR376B:
5'(6-FAM)- TGCACTGGATACGACACGTGGA-(TAMRA-Sp)3'.

2.9 Indirect Immunofluorescence

Stable monoclines treated with H₂O₂, and after the treatment finished, the cells washed with 1X PBS (2ml/well). 3.7% PFA was used to fixate the cells for 20min RT dark. The fixative removed, and each well was washed with 5ml PBS(1X) 6 times in every 10 minutes. Then, blocking was carried out with 0,1% BSA+0.1% saponin in 1X PBS for 30 min at 4°C on the shaker. After the blocking solution removed, the cells incubated in primary antibody; H2AX-P (Milipore-05-636, 1:250) or P62 (Abnova, H0008878, 1:250) for 1 hour at RT on shaker. After the incubation completed, the cells washed with 1X PBS for 2 times. Incubation with secondary antibody Alexa Fluor 568 mouse (Invitrogen, A11004, 1:500) for H2AX-P and P62 was carried out for 1 hour at RT on shaker. Then, the cells washed with 1X PBS, and Hoescht (Invitrogen, H3570, final concentration:1µg/ml) can be used to stain the nuclei if necessary for 10 min, and after staining 2 times 1X PBS wash was carried out. The slides were mounted and inspected under 60X magnification using Zeiss LSM Confocal microscope.

2.10 DCFDA and Mitotracker Red Analysis

30 min DCFDA (Invitrogen, D399) staining (1µM) or 15 min Mitotracker Red staining (Invitrogen, M7512) (50 nM) was carried out in DMEM +/+/+ for FACS or in PBS+FBS mix for microscopy analysis in dark 37°C and after PBS wash, cells were analyzed with BD FACS Canto flow cytometer or cells were fixed in 3.7% formaldehyde for 20 min, washed with PBS, mounted and inspected under under 60X magnification using Zeiss LSM Confocal microscope.

2.11 Cell cycle analysis

Cells seeded at the 12 well plate 24 h before experiments. Then cell trypsinized and fixed with 70 % Ethanol overnight. Then cells treated with RNase (100 µg/ml) and PI (40 µg/ml, P3566, Invitrogen, Eugene, OR).

Samples were then incubated for 10 min in the dark on ice. FACScan analyses of PI and FITC positivity were performed in a BD FACS Canto Flow Cytometry System (BD, San Jose, CA) and analyzed with using FlowJo Program. At least 10,000 events analyzed per experimental point.

2.12 Scratch Assay

Cell migration determined by wound-healing assays. Briefly, cells were cultured in 6-well plates (5×10^4 cells per well) for 24 h. These cultures were then scratched using a sterile plastic micropipette tip to create an artificial wound. Photographs were taken 24 and 48 h later using a BX70 inverted microscope (Olympus, Tokyo, Japan).

2.13 Transwell Migration Assay

Transwell migration assay performed in 24-well chambers with 8 μ m pores (BD falcon, Bedford, MA, USA). 100,000 plated into the upper chambers in medium containing no FBS, and lower chambers filled with culture media containing 10% FBS as a chemo-attractant. The chambers were incubated at 37°C for 16 hours, after which, non-invading cells removed with a cotton swab. Successfully invaded cells were fixed by methanol for 15 minutes, stained with 0.1% crystal violet for 15 minutes, and counted under a light microscope.

2.14 Matrigel invasion assay

Matrigel invasion assay performed in 24-well matrigel coated chambers with 8 μ m pores (BD falcon, Bedford, MA, USA). 100,000 plated into the upper chambers in medium containing no FBS, and lower chambers filled with culture media containing 10% FBS as a chemo-attractant. The chambers were incubated at 37°C for 16 hours, after which, non-invading cells removed with a cotton swab.

Successfully invaded cells were fixed by methanol for 15 minutes, stained with 0.1% crystal violet for 15 minutes, and counted under a light microscope.

2.15 Colony formation assay

Stable cells trypsinized and 125 cell/well, 250cell/well, or 500cell/well were seeded in 6-well plate and left to grow for 14 days (media change in every 3 days). The colonies that obtained at the end of 2 weeks visualized under a light microscope. The media discarded, and after 1X PBS wash, 5minute MetOH 100 % fixation at RT on shaker was carried out. After fixation, crystal violet (0,5%) staining was carried out for 10 min RT on shaker. After staining, extensive PBS wash was carried out to get rid of the excess dye and plates were left to dry. Image J analysis was carried out to quantify the number of colonies.

2.16 Nude mice xenograft models

Huh7 HCC cells mice xenografts usually done in athymic nude mice. Control and MIR376B stable cells (10 million cells) resuspended in 100µl matrigel (BD,356234) and administration of cells+matrigel to mice was carried out in the cold (on ice). Subcutaneous injection of tumor cells to left flank region of in each 4 mice/group was carried out. Ethical permission taken for this part of the project from Bogazici University Ethical Board. These experiments were carried out in collaboration with Arzu Temizyürek from Bogazici University.

2.17 *Ex vivo* tissue protein analysis

Frozen tissue (present in liquid nitrogen) was broken into pieces, weighted, and washed in lysis buffer. After spinning at 13,000 rpm for 1 min at 4 °C, 2.5 µl/mg RIPA was added. Using a disposable tissue grinder, pieces were ground in time with approx. 40 strokes. Homogenization performed on ice by spinning at 13,000 rpm for 15 min at 4 °C.

2.18 TUNEL Assay

Ex vivo, tissues cut at 4-micron size and fixed on thick glass. In Situ Cell Death Detection Kit were used (11684795910, Roche) and experiments conducted upon manufacturer instructions on our tissue slides. DAPI used as a counterstain of nuclei.

2.19 Statistical analyses

Statistical analyses were performed using Student's two-tailed t-test. Data represented as means of $\pm$ S.D. of n independent experiments. Values of $p < 0.05$ considered as significant.

2.20 Ethic Statement

Samples collection from patients approved by Çukurova University Medical Faculty Ethics Committee and written informed consent obtained from all participants.

3 RESULTS

3.1 Autophagy deficiency in *MIR376B* stable HCC clones

To create a model for *MIR376B* deregulation in cancer, stable clones that overexpress *MIR376B* generated in Huh7 liver cancer cell lines. Confirmation of the overexpression Taq-Man qPCR analysis for these monoclonal clones carried out (**Figure 3.1 A**). Compared to controls, *MIR376B* levels of all positive clones found almost 3 to 5 fold higher. We then further characterize the monoclonal clones. qPCR analysis utilized to validate the *MIR376B* target levels in Huh7 cells. Targets significantly decreased in M1 and M2, yet decrease was not significant in M3 monoclonal clone in both genes (**Figure 3.1 B and C**). Therefore, we decided to further run our experiments with C1, C2, and M1, M2 clones. In addition to mRNA level, target protein levels of ATG4C and BECN1 evaluated by western blot analysis (**Figure 3.1 D-G**).

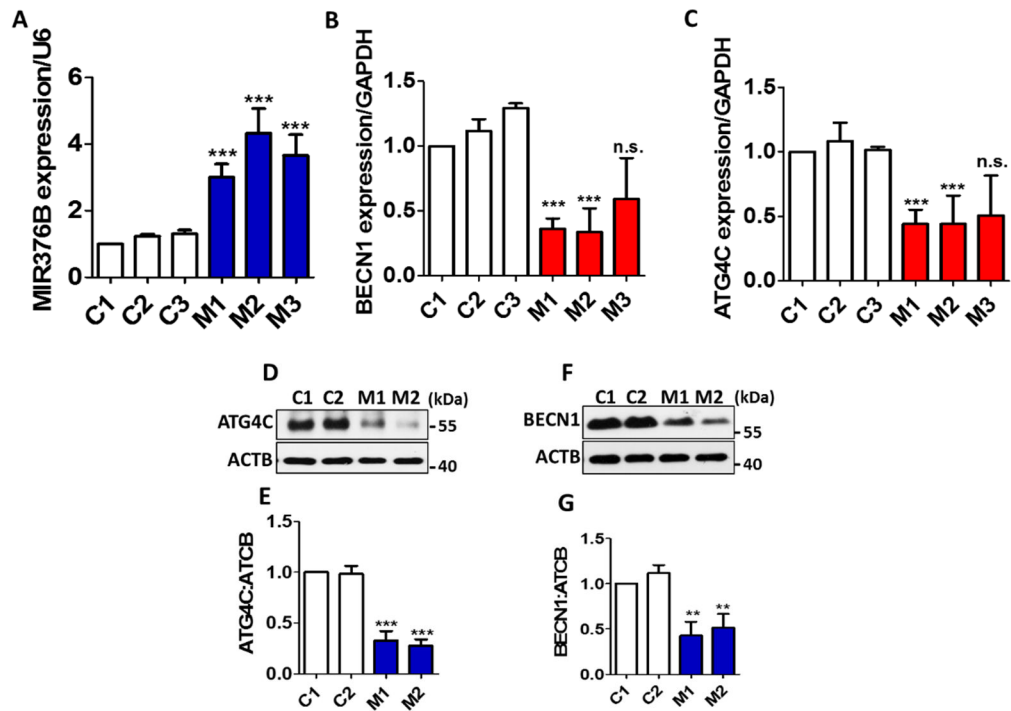


Figure 3. 1 Characterization of the Huh7 *MIR376B* clones. **A.** Taq-Man qPCR analysis conducted and miRNA level evaluated. **B and C** Target level of *MIR376B* analyzed by Syber-green qPCR. **D-F** Target level of *MIR376B* analyzed by western blotting to further confirmed clones represents the expected function in HCC cells (Control clones: C1, C2, C3; *MIR376B* Clones: M1, M2, M3). (mean±SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001)

Furthermore, we analyzed the autophagic capacity of the monoclonal cells. Initially, we performed the LC3 shift assay to validate the attenuation of autophagy. EBSS used as an autophagy inducer. Besides, to evaluate autophagic flux, we further treat the cells 1 h before experiments with E64/PepA, which both block the degradation of autophagic cargo by enabling protease activity in lysosome.

According to LC3 shift assay, under basal condition, *MIR376B* monoclonal cells (M1 and M2) exhibit autophagic attenuation. GFP-LC3 dot counting in monoclonal cells also support the immunoblotting (Figure 3.2 A-C). 4 h EBSS significantly induce autophagy in control Huh7 cells. In line with the basal autophagy, *MIR376B* also blocks starvation-induced autophagy (Figure 3.2 D-F). According to our data, *MIR376B* overexpressors manifest strong autophagic attenuation according to both methods. In line with the EBSS stimuli, autophagic flux in *MIR376B* monoclonal cells is not functioning (Figure 3.2 G-I).

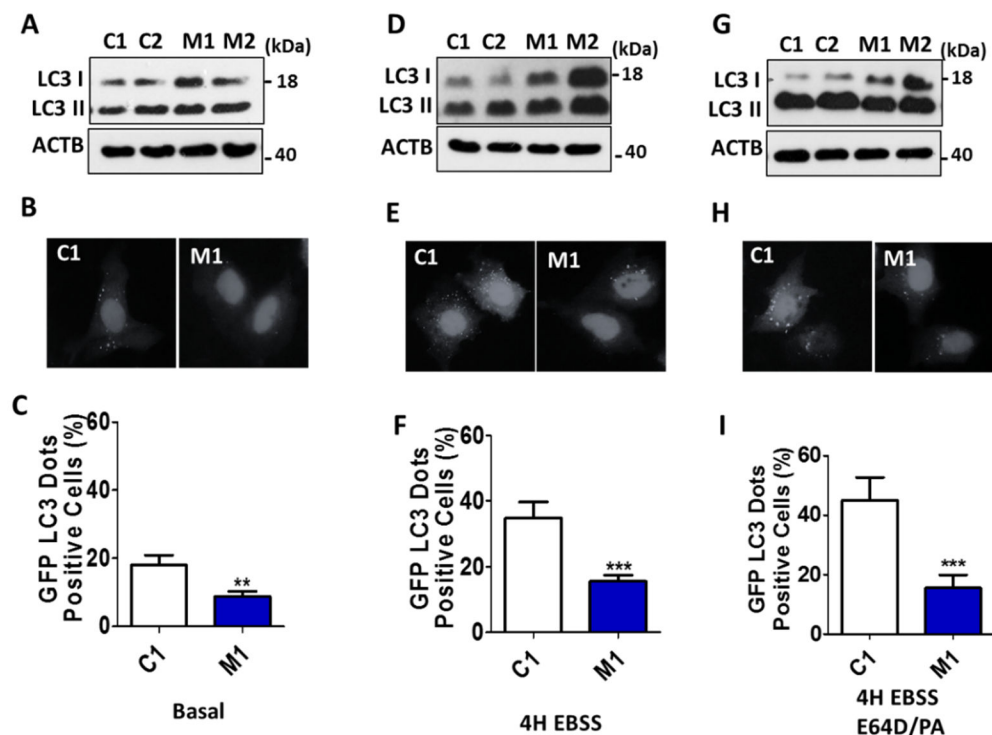


Figure 3. 2 Characterization of the autophagic capacity of *MIR376B* clones by LC3 dependent analysis. A, D, G. LC3 shift analyzed by western blotting in *MIR376B* clones. EBSS (4 h) and E64D/PA used for flux control. Actin used as a loading control. B, E, H. Representative microscopy images of Huh7 monoclonal cells. C, F and I. Graphs represent average GFP-LC3 dot counting of at least 150 cells. (mean±SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001) (C1, control; M1, *MIR376B* overexpressed monoclonal cells)

Autophagic activation does not necessarily finalize with the degradation of the cargo. On the other hand, with previous experiments, we could not fully understand which stage of autophagy affected upon *MIR376B* overexpression. Thus we performed cargo degradation assay by immunoblotting to characterize functional autophagy. Besides, we also performed immunostaining to evaluate the cargo accumulation in the cytosol.

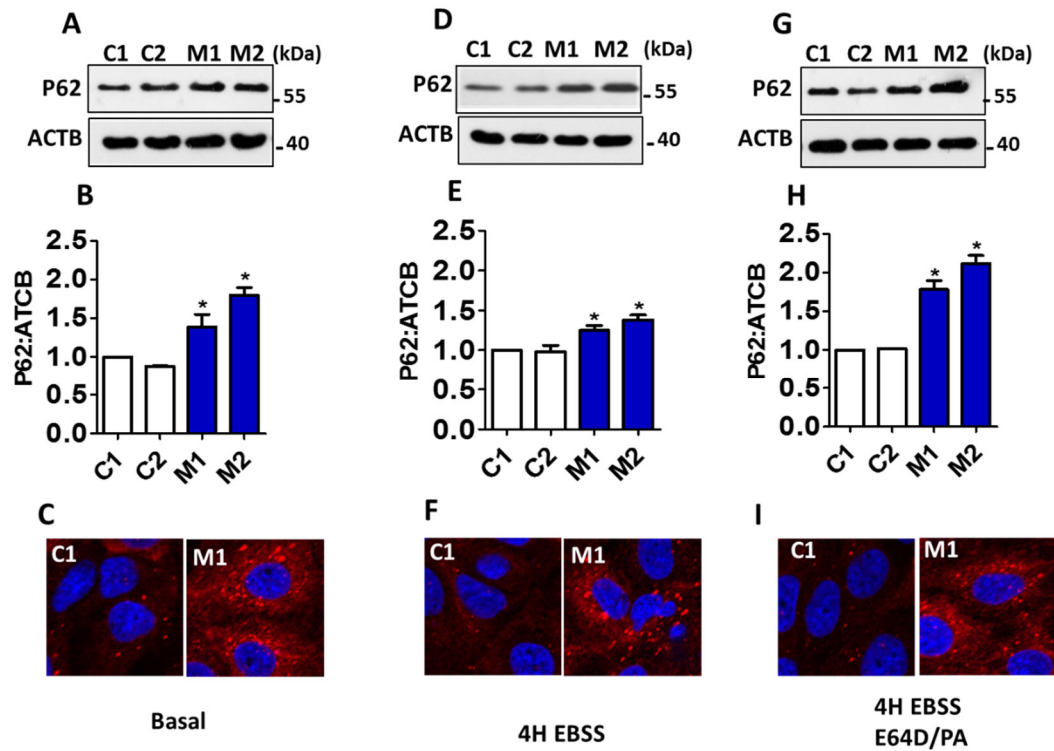


Figure 3.3 Characterization of the autophagic capacity of *MIR376B* clones by cargo degradation assays. A, D, G. p62 cargo degradation analyzed by western blotting in *MIR376B* clones. EBSS (4 h) and E64D/PA used for flux control. Actin used as a loading control. B, E, H. Band intensity analysis of p62, which are corrected according to control actin. Representative microscopy images of Huh7 monoclonal cells. (mean±SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001) C, F and I. Representative microscopy image of p62 accumulation. (C1, control; M1, *MIR376B* overexpressed monoclonal cells)

According to data, the accumulation of p62 protein was prominent in *MIR376B* clones compared to control clones validated by two different methods in HCC cells (**Figure 3.3 A-C**). Upon EBSS treatment, level of p62 accumulation diminished in control cells, whereas there was no noticeable change in *MIR376B* clones (**Figure 3.3 D-F**). Activation of autophagy through starvation in Huh7 cells observed in control clones (LC3-II accumulation and p62 degradation upon starvation). Autophagic flux with the blockage of lysosomal protease activity by E64D and Pepstatin A also checked. Data suggest that autophagic flux are function usually in control clones, yet inhibitors did not alter the autophagy in *MIR376B* clones (**Figure 3.3G-I**).

Therefore, we proved that overexpression of *MIR376B* resulted in autophagic attenuation in HCC both activation and degradation process.

3.2 HCC cell proliferation in autophagy-attenuated *MIR376B* clones

To observe the growth difference between control and *MIR376B* overexpressing clones, short term growth analysis (24h-96h) carried out with several different methods. First trypan blue cell counting assay performed. Data showed that *MIR376B* clones exhibit attenuated proliferation ratio in comparison to control cells (**Fig 3.4 A**). In parallel, *MIR376B* clones could not close the wound in the scratch assay, whereas control clones did more prominently (**Figure 3.4 B and C**). As a result of this analysis, we observed that the growth of *MIR376B* clones was slower compared to control clones in the short term.

In the long term assay, colony formation ability checked. Definite increase in the level of colonies for *MIR376B* clones is seen compared to control clones showing that chronic autophagy attenuation leads to increased proliferation and growth ability of these clones in Huh7 cell lines (**Figure 3.4D and E**). Of not, colonies from *MIR376B* plates exert different character then control territories. They seem bigger in diameter than control clones.

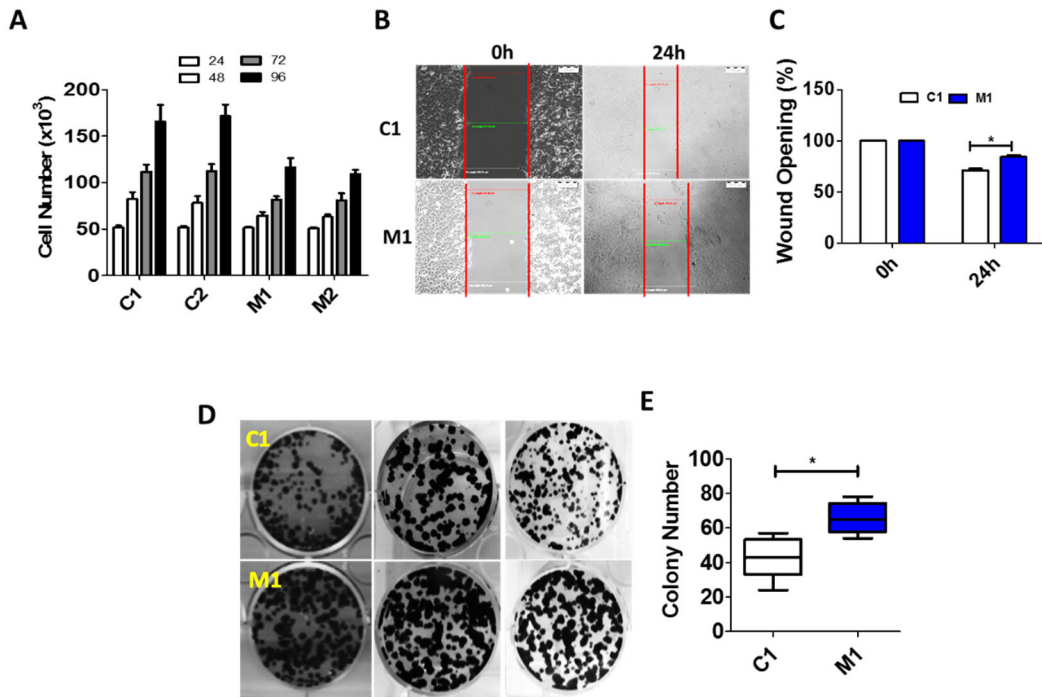


Figure 3. 4 Short and Long term growth analysis of the HCC clones. **A.** Trypan blue exclusion assay utilized in time-dependent manner. **B.** Scratch assay and assessing wound healing capacity of the clones checked. **C.** Long term growth analysis evaluate by the formation of the colonies. **D.** Graphical representation of colony number in monoclonal. (mean±SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001). (C1, control; M1, *MIR376B* overexpressed monoclonal)

3.3 HCC cell cycle and cell size in autophagy-attenuated *MIR376B* clones

Autophagic attenuation may alter cell size. Thus, we also analyzed the size change in these cells. We first performed FACS analysis. Cell size and granularity affect FACS distribution of the cells. According to our data, *MIR376B* clones were more prominent in size and higher in granularity, within quadrant 3 (**Figure 3.5 A and B**). Moreover, cell cycle distributions of clones also affected autophagic attenuation. *MIR376B* monoclonal exert a high level of G2/M arrest in comparison with control counterparts (**Figure 3.5 C and D**).

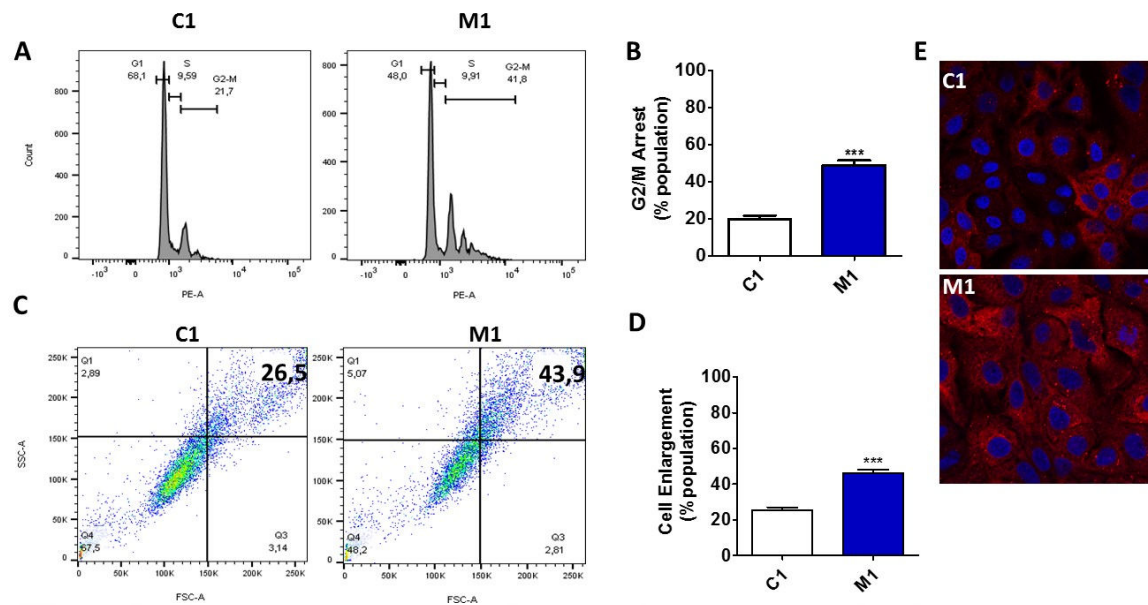


Figure 3.5 Cell cycle and size analysis of *MIR376B* clones. **A and B** FACS Cell cycle distribution graph of FACS **B**. Graphical quantification of *MIR376B*-derive cell enlargement. **C and D**. Cell size and granularity analysis of clones. **E**. Representative image of cells (mean±SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001). (C1, control; M1, *MIR376B* overexpressed monoclones)

3.4 Oxidative and genotoxic stress in *MIR376B* clones

So far, we validate that our *MIR376B* clones showed potent autophagic inhibition and also have altered proliferation. In order to understand the proliferative switch in *MIR376B* clones, we analyzed several critical phenomena, including ROS and DNA damage. In line with this, we first performed DCFDA staining under basal. Surprisingly, *MIR376B* clones accumulate a significant amount of ROS in especially around mitochondria (**Figure 3.6 A and B**). N-acetyl cysteine, NAC, used as a ROS scavenger in our experiments. Fluorometer analysis has been utilized to detect fluorescein upon ROS accumulation.

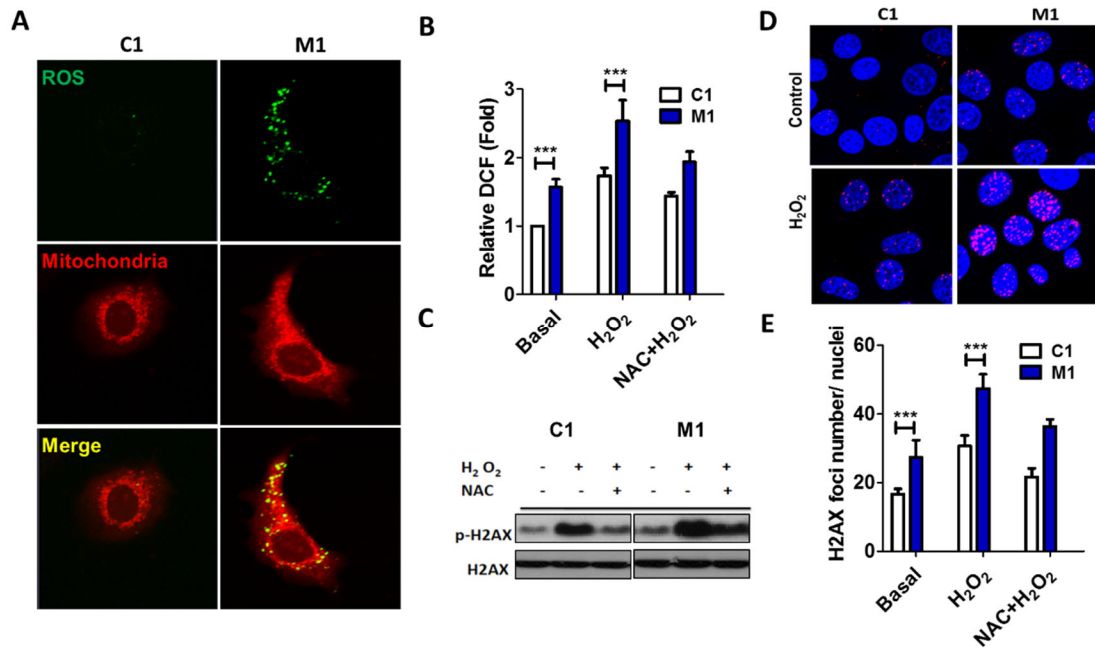


Figure 3.6 Oxidative stress and DNA damage analysis of the *MIR376B* clones. A and B. Huh7 clones examined upon basal condition. Hydrogen peroxide- used as a positive inducer in cells were both stained with DCFDA and Mito-Tracker Red and samples inspected by confocal microscopy. **B.** Huh7 clones were evaluated under the same condition by the fluorometric reading of Fluorescein. **C.** Immunoblotting of gamma-H2Ax upon stimuli and **D and E.** assessing of DNA lesions by microscopy and **E.** graph representation of DNA damage. (mean±SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001). (C1, control; M1, *MIR376B* overexpressed monoclonal clones)

Beside to ROS accumulation, *MIR376B* clones showed strong positivity according to gamma-H2AX staining, which is the marker of DNA lesions. Moreover, an acute dose of hydrogen peroxide increases the foci formation more prominently in *MIR376B* clones than the control clone in both microscopy and immunoblot analysis (**Figure 3.6 D and E**). Data suggest that autophagic attenuation increased the ROS accumulate and make the cell more susceptible to DNA damage. Of note, ROS and DNA damage accumulation is also known as one of the critical HCC drivers through inducing genomic instability.

3.5 HCC cancer invasion and migration in *MIR376B* clones

To evaluate the oncogenic properties of *MIR376B* on HCC cells, we utilized the transwell migration and invasion assay.

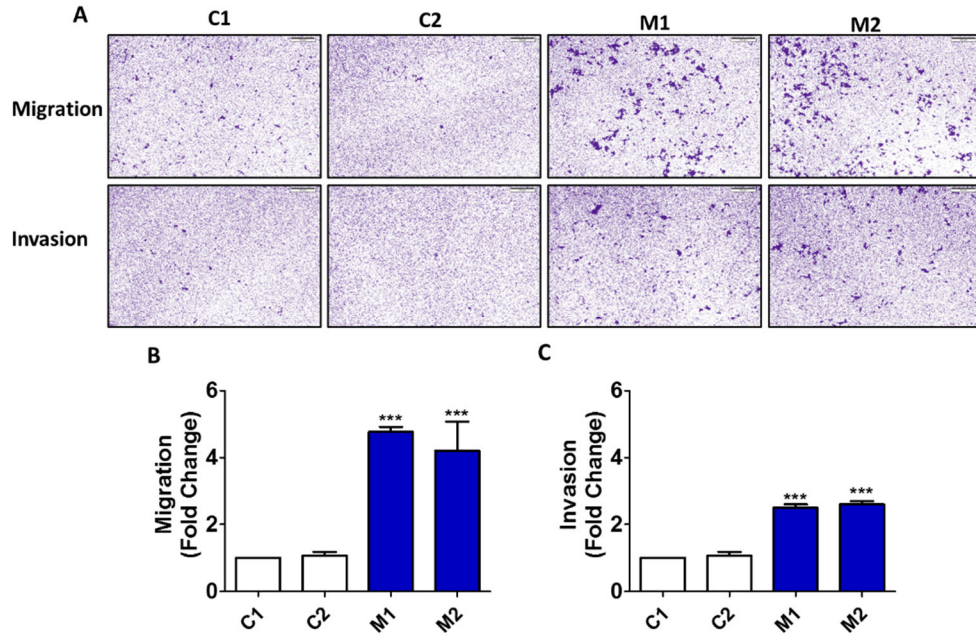


Figure 3. 7 Migration and Invasion analysis A-C. Cell migration determined by transwell migration assay. Cell invasion utilized by matrigel invasion assay in Huh7 liver cancer cell line. (mean $\pm$ SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001). (C1, control; M1, *MIR376B* overexpressed monoclonal)

MIR376B effect both HCC cell migration and invasion *in vitro*. Both cell migration and invasion significantly increased almost 3 to 5-fold range in comparison to control HCC cells. Our results suggest that autophagic attenuation in HCC may facilitate tumor in migration and invasion, which further develop metastasis (**Figure 3.7 A-C**).

3.6 HCC tumorigenesis in *MIR376B* clones

To assess the tumorigenic capacity of *MIR376B* overexpressing cells, cells injected subcutaneously into nude mice. The results indicated that the size of the tumor increased significantly compared to control tumors, leading to the conclusion that chronic autophagy blockage may result in higher growth in tumors (**Figure 3. 8 A and B**).

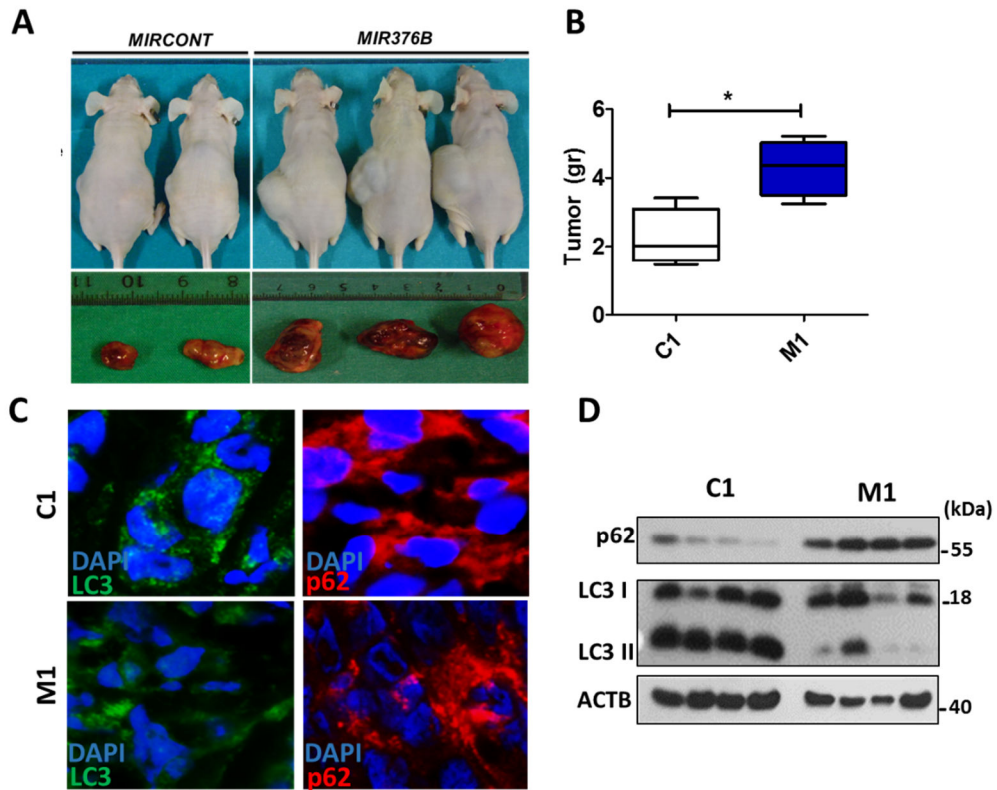


Figure 3. 8 HCC xenograft and frozen tissue section immunofluorescent analysis. **A.** Both *MIR376B* and control clones subcutaneously injected to the left flanked region of the mice, and then after 2-month mice sacrificed, tumors were removed and pictured. **B.** Removed tumors measured with fine-scale and then graphs drawn. **C.** *Ex vivo* tumor tissues evaluated by their autophagic capacity by immunofluorescent analysis. **D.** *Ex vivo* tissue proteins analyzed by autophagic autophagy capacity (mean $\pm$ SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001). (C1, control; M1, *MIR376B* overexpressed monoclonal)

We further examine the *ex vivo* tumors tissues, to validate *MIR376B* overexpression and target downregulation. Autophagy in the tumor found to be attenuated. Increase in both LC3 shift and autophagosome staining in tumor tissues alongside with P62 accumulation suggest that *MIR376B*-suppress autophagy even in vitro conditions. (Figure 8C and D).

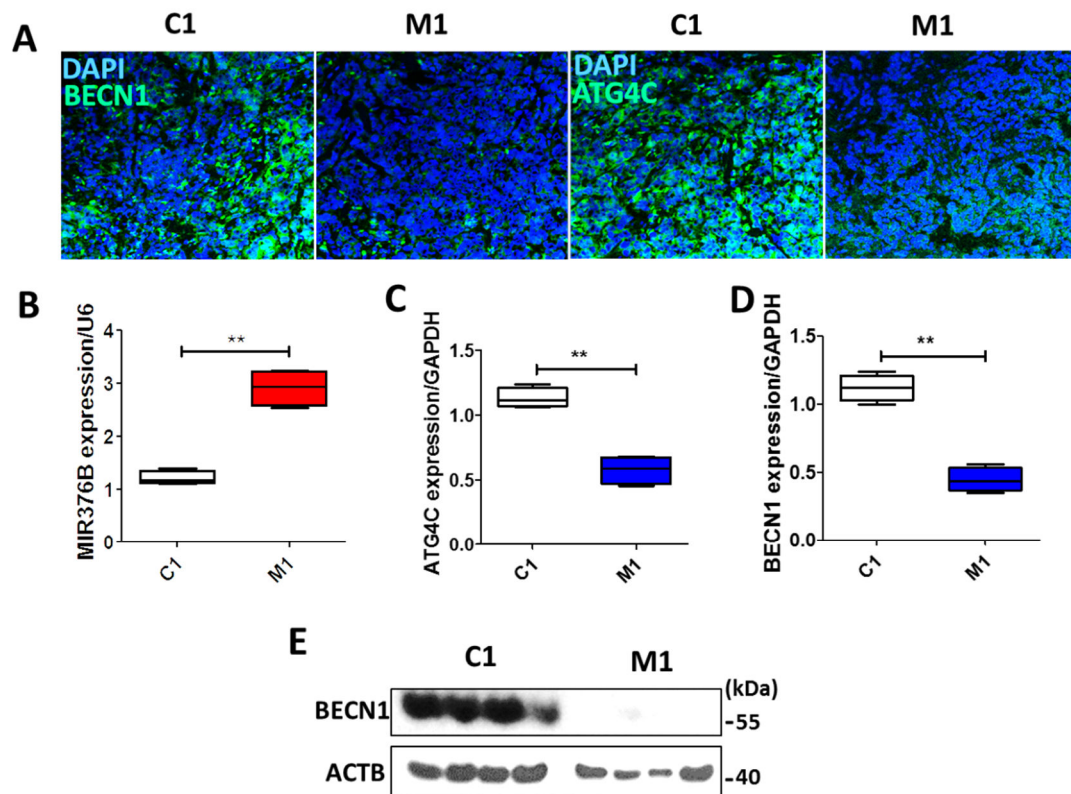


Figure 3.9 *Ex vivo* target and frozen tissue section immunofluorescent analysis of tumors **A.** Immunofluorescent target analysis. **B, C and D;** *ex vivo* miRNA and the target status. **E.** Immunoblotting of BECN1. (mean±SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001). (C1, control; M1, *MIR376B* overexpressed monoclonal)

To further support our hypothesis, we also evaluate the *MIR376B* and target gene levels in parallel. *Ex vivo* tumor tissues exhibit higher *MIR376B* and decreased targets level (Figure 3.9 A). In parallel, we also checked the level of target mRNAs too. We detect a clear decrease in both the target mRNA level in *ex vivo* tumor tissues (Figure 3.9 C and D). Moreover, the protein level of BECN1 almost disappeared upon *MIR376B* overexpression (Figure 3.9 E).

Besides, autophagy, we further analyzed the tissues, and we performed TUNNEL staining. TUNNEL positivity is one of the crucial test to determine DNA damage in *ex vivo* tissues. According to our results, we did not notice a significant change in TUNNEL positivity in MIR376B clones in comparison with control ones. Of note, we used Micrococcal DNase I for positive control and following treatment significantly increased the level of GFP signal, which reflects DNA damage *ex vivo* (**Figure 3.10 A and B**).

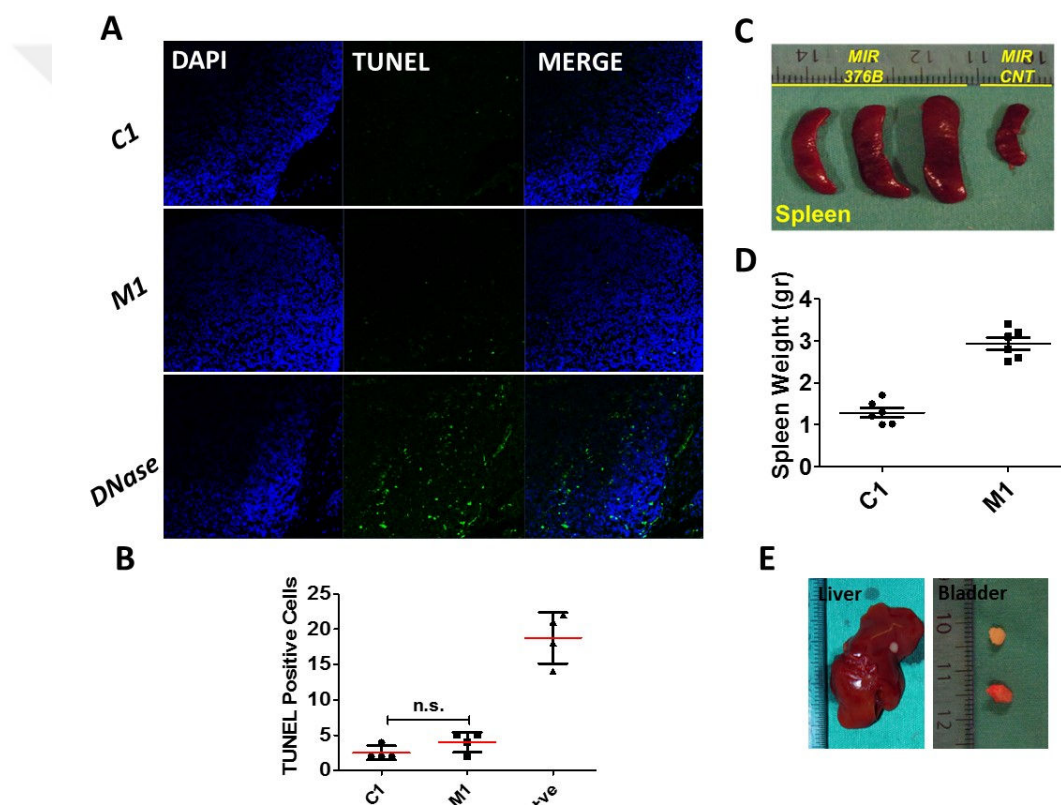


Figure 3. 10 Autophagy-independent changes analysis of tumor A and B. TUNNEL analysis by IF. **C and D**; *ex vivo* spleen analysis **E**. *Ex vivo* metastasis analysis (mean±SD of independent experiments, n=3, *: p < 0.05; **: p < 0.01; ***: p < 0.001). (C1, control; M1, *MIR376B* overexpressed monoclonal)

On the other hand, we also noticed splenomegaly in *MIR376B* clone injected mice (**Figure 3.10 C and D**). Surprisingly, we notice distant organs metastases in two different mice that we inject *MIR376B* clone (**Figure 3.10 E**). Although we could not fully dissect the whole animal, these metastases were prominent and it's correlated with our *in vitro* migration and invasion data.

3.7 *MIR376B* in HCC

Until now, we characterized our autophagy attenuated clones both *in vitro* and *in vivo* experimental conditions. We also would like to evaluate the correlation of miRNA and its' targets in patient tissues. Thus, we obtained HCC patient tissues from Çukurova University hospital. Then, we isolated RNA and analyzed the correlation between *MIR376B* and BECN1. According to our findings, *MIR376B* upregulated in 35 % of total HCCs (**Figure 3.11 A and B**). Unfortunately, we could not obtain significant data about the correlation of *MIR376B* and BECN1 in patient tissue (**Figure 3.11 C**). Although our data has in some extension through the negative correlation which has p value =0,0542.

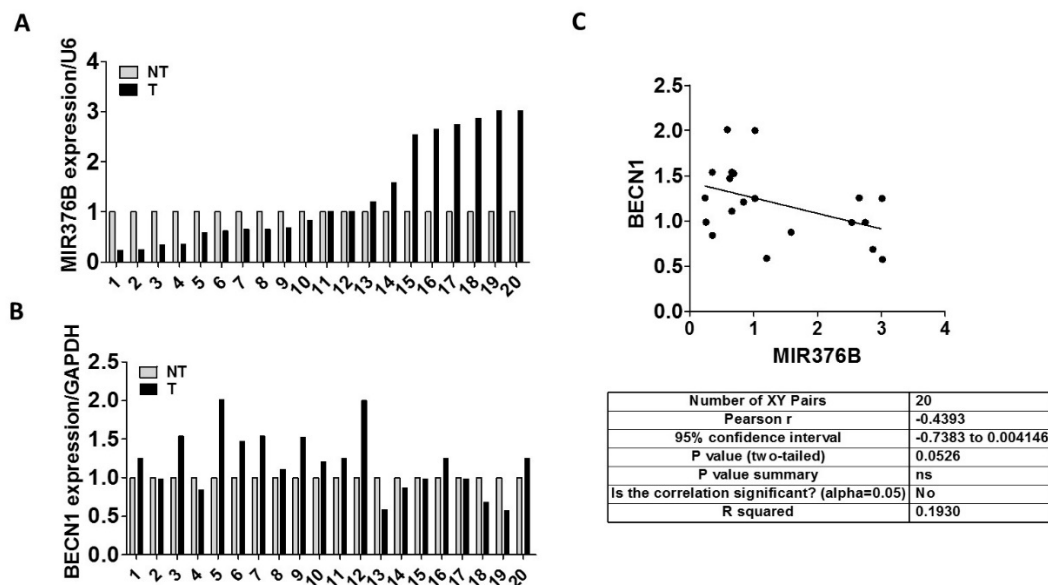
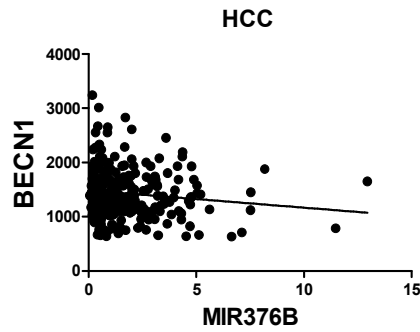


Figure 3. 11 Expression analysis of *MIR376B* and BECN1. A and B expression analysis of patient tissue pairs: tumors (T) and non-tumors (NT). C. Correlation analysis of expressions (Pearson correlation analysis).

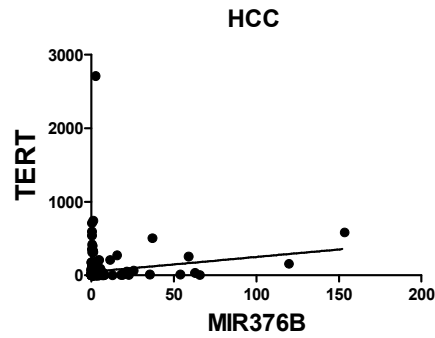
Because of the limitation, we also checked *MIR376B* and BECN1 co-expressions through bioinformatic analysis tools (<http://www.oncolnc.org>). We subtract the *MIR376B*, and BECN1 expressions data belongs to 293 patients tissue from LIHC section upon our request. Strikingly, we found a significant negative correlation between BECN1 and *MIR376B* expression in HCC patients (**Figure 3.12**).



Number of XY Pairs	293
Pearson r	-0.1240
95% confidence interval	-0.2352 to -0.009493
P value (two-tailed)	0.0339
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.01537

Figure 3. 12 Correlation analysis of *MIR376B* and *BECN1* *in silico* Correlation analysis of expressions (Pearson correlation analysis). (<http://www.oncolnc.org>)

Above mentioned correlation data showed the importance of the *MIR376B* and its role through *BECN1* in HCC. More than that we also would like to investigate other correlations that might be crucial for hepatocarcinogenesis. For instance, TERT activation which is established as one of the main driver of hepatocarcinogenesis. According to our analysis there is no available publication about the *MIR376B* level and TERT activation. On the other hand, TERT also did not identified as an target gene for *MIR376B*. Thus, we performed the same *in silico* analysis for *MIR376B* and TERT (**Figure 3.13**).



Number of XY Pairs	294
Pearson r	0.1478
95% confidence interval	0.03400 to 0.2579
P value (two-tailed)	0.0111
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.02186

Figure 3. 13 Correlation analysis of *MIR376B* and TERT *in silico*. Correlation analysis of expressions (Pearson correlation analysis). (<http://www.oncolnc.org>).

According to our analysis, we found a significant positive correlation between TERT and *MIR376B* expression in HCC patients (**Figure 3.13**). This is the first time that we showed *MIR376B* may have role in TERT activation in HCC.

4 DISCUSSION

Autophagy-cancer connection is a relatively new area of research. A context-dependent link exists between autophagy and cancer: In the early stages of carcinogenesis and cancer initiation, a tumor-suppressor attributed to autophagy. Indeed, autophagy is responsible for the elimination of organelles such as mitochondria, control of reactive oxygen release and protection from DNA damage, preserving genomic stability. In established tumors and especially in solid tumors with oncogenic K-RAS pathway activation, autophagy was shown to contribute cancer growth, allowing resistance to unfavorable conditions that occur in some tumor regions due to inefficient tumor vascularization (i.e. lack or low levels of nutrient supply and oxygen in spite of high metabolic requirements)

Above-cited studies underline the importance of autophagy for health and disease in the liver. In particular, with the advance of studies on autophagy cancer, the role of autophagy in HCC development and management becomes more apparent. Primarily, reviews on the contribution of autophagy and related mechanisms to HCC chemoresistance is of particular interest. There are several studies correlating autophagic activity with resistance to chemotherapeutic agents, including sorafenib. Several independent labs are currently working on the discovery of novel small molecules that will be capable of manipulating autophagy for treatment purposes. Further studies, including clinical studies, are required to fully reveal the potential of above-mentioned strategies and these potential new drugs, alone or in combination with classical drugs, for the treatment of liver diseases and HCC.

Moreover, a strong connection between autophagy and liver pathologies, including NASH, HBV, and HCV infection and cirrhosis reported too. For instance, autophagy constitutes a significant clearance of mechanism for intracellular pathogens, such as viruses. But, some viruses, including HBV and HCC might hijack autophagic membranes during their replication. Additionally, several autophagy-deficient animal models were shown to suffer from hepatic steatosis, and independent studies consistently demonstrated that autophagy is involved in lipid and glycogen metabolism. These and other abnormalities of liver function and pathologies are also closely related to HCC development.

There are several approaches mentioned in the context of cancer and autophagy. Autophagy might play several different roles in the cell and the context-dependent manner in cancer. In order to dissect the role of autophagy, we have to assess the problem from different perspectives.

Investigating tumor cell autophagy is belongs to one of those approaches. Several genetic modifications made in mice by knocking out autophagic genes which then follow the disruption of autophagic activity. Several studies showed that blocking the ability of autophagy leads to the accumulation of ROS, DNA damage, and increased inflammation and trigger the formation of carcinogenesis. Thus, autophagy seems to act as a tumor-suppressive mechanism during the early stage of tumorigenesis by eliminating tumor-promoting factors, including ROS [195, 256, 257]. Of note, ROS and subsequent tissue damage and inflammation are one of the well-known underlying tissue damage factors in the liver where all those factors further associated with hepatocarcinogenesis. However, those tumors arise from autophagic ablation mostly remain benign. In line with this, most of the cancer cells have been associated with high autophagic activity to support their growth, survival, and malignancy. Even though autophagic ablation prepare favorable condition for carcinogenesis, yet autophagic activity seems to required for cancer progression.

As a catabolic process, autophagy supply cellular building blocks to cells and maintain cellular homeostasis. In line with this, several reports showed that cancer cells exert elevated cellular autophagy to support high metabolic demand of cancer cells in comparison to the healthy cells[193, 201, 258]. Thus a deficiency in autophagy may suggest therapeutic aspect. For instance, conditional whole-body deletion of Atg7 in established KRAS-driven lung cancer bare mice showed prominent anti-tumor activity [259]. Therefore, cancer cells seem to exert a more sensitive character than healthy cells upon autophagy deficiency where might support a therapeutic approach to treat cancer.

In all above-mentioned conditions, predominantly discussed the tumor cell autophagy in cancer. On the other hand, there are several other phenomena have been investigated in the cancer field, including tumor microenvironment. The tumor microenvironment is a complex term which consists of several cellular and acellular components. Fibroblasts, endothelial cells, and immune cells are the most established tumor microenvironment-associated cellular components. In line with this, acellular components consist of extracellular matrix components such as collagens, matrix metalloproteinases, and also glycans. In the last decade, several reports published that almost all of these components affects tumor growth and characteristics. Thus, understanding the role of autophagy in this context is quite remarkable. Host autophagy generally reported promoting tumor growth. For instance, several tumors, including KRAS mutant and Trp53-/-driven lung and pancreatic tumors found to enter regression upon the attenuation of autophagy [259, 260]. More than that, autophagy competent tumors where allografted onto either autophagy-wild type or autophagy-deficient mice and results support that host autophagy-deficiency in host affect tumor growth in several tumor types[261]. More than that, the autophagic capacity of tumor stroma is also found to crucial for the growth of pancreatic adenocarcinoma[262]. According to literature, autophagy has been addressed to support biosynthetic pathways building blocks, especially amino acids to the cancer cells for maintaining cell survival. Host autophagy was found to sustain the level of circulating arginine, which supports cancer growth[261]. In line with this, autophagy in pancreatic tumor cells induces the secretion of alanine, which is responsible for the growth of tumor [262]. Thus, both circulatory and tumor microenvironment-derived factors affect autophagy in tumor cells. Cells which reside in the microenvironment are the primary supplier of nutrients.

Besides these, tumor microenvironments are also crucial for tumor cell fate, especially in metastasis. In as much as the tumor microenvironment, autophagy is also essential in tumor metastasis. Epithelial characterized as tightly bound cells, and they are lack of mobility. EMT is one of the processes that help epithelial cells to gain mobile characteristics. Besides the changes happen in tumor cells, differentiation or reorganization of ECM also shoed to affect cellular migration.

Autophagy and tumor microenvironment orchestrate both of these procedures. Several studies showed that autophagy supports tumor cell survival in the bloodstream especially help cells to resist anoikis which is one of the characterized cell death mechanism, often manifested upon cellular-adhesion lost [263]. In addition to this, as autophagy is a stress response mechanism, metastatic cells need to adapt itself to this new environment [264]. Autophagic activity helps tumor cells to suit this foreign environment.

Autophagy in stromal cells supports alternative energy sources rather than amino acids such as free fatty acid, lactate, and ketone bodies, which then used for TCA cycle and glycolysis for tumor cell survival. For instance, Cav-1 is one of the essential endosomal pathway-related proteins. Autophagy degrades Cav-1 in stromal cells, in Cav-1^{-/-} stromal cells exert enhanced glycolysis through upregulation of the glycolytic enzymes like pyruvate kinase [265]. In addition to the nutrient supply, more likely stromal cells also secrete ECM components such as collagen and facilitate the re-organization of ECM. Surprisingly, there is a report showed that collagen VI upregulation specifically in breast cancer patients correlated with lymph node metastasis [266].

MiRNAs identified as exosomal delivered molecules which shared between cancer cells and its stroma. There are several reports showed that stromal-derived exosomal miRNAs affect tumor cell survival. For instance, MIR96-5p level increased in fibrotic liver tissues. Moreover, this specific miRNA cause decrease in α -SMA and collagen, which are ECM-related proteins in stromal cells. Surprisingly this particular miRNA targets ATG7, which is a crucial autophagy protein and re-introduction of ATG7 rescue the phenotype [246].

Lack of knowledge to the treatment of the metastasis is one of the emerged problems in the field of cancer. Several studies have addressed the role of autophagy in metastasis-related cellular pathways, including cell migration, invasion, maintenance of stem cell phenotype, resistance to anoikis, etc. Therefore, understanding the role of autophagy also essential to approach HCC metastasis. A better understanding of autophagy mechanisms and its abnormalities and its connection to liver diseases and disease-causing factors will certainly improve current diagnosis, treatment, follow-up, and prevention strategies concerning HCC.

As we mentioned above, miRNAs as a regulator of the autophagy may serve a crucial role to investigate the regulation of the autophagy in cancer. In our hand, *MIR376B* implicated in autophagy regulation. Therefore, we studied autophagy-cancer connection and discussed the role of *MIR376B* in hepatocellular carcinoma concept. *MIR376* family members exerted their controls on autophagy through their direct binding to the 3' UTR sequences of the messenger RNAs of two essential autophagy proteins, BECN1 and ATG4C. Indeed, both mRNA and protein levels of BECN1 and ATG4C decreased following overexpression of the miRNAs.

Deregulation of *MIR376* family members observed in various cancer types. Although some studies conducted on HCC, they either evaluate the level of the *MIR376B* in HCC specimen or serum. Therefore, investigating the role of the *MIR376B* HCC might provide us a novel and sophisticated approach to cure cancer. Since autophagy activation is vital for some rapidly growing solid tumors, *MIR376* family levels may be a critical factor for cancer cell survival and growth.

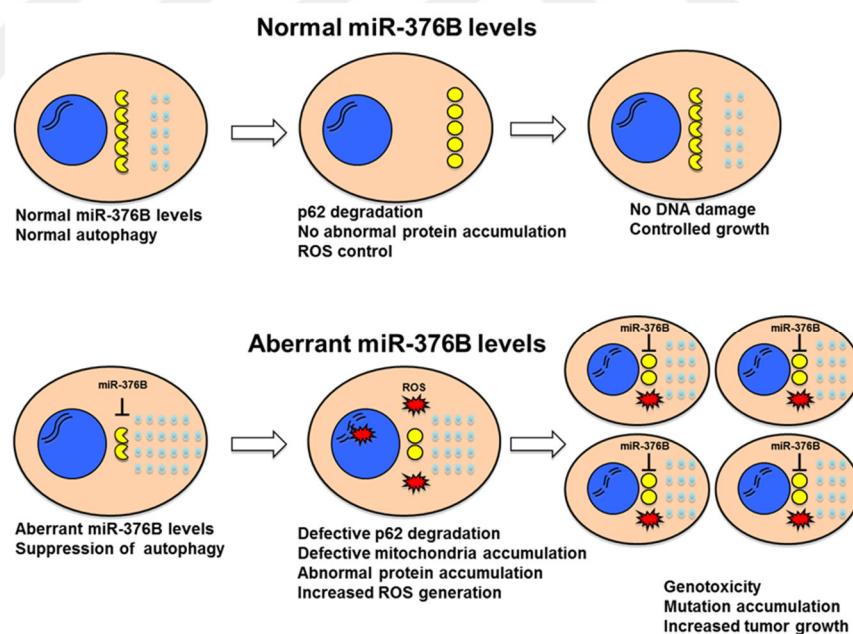


Figure 4. 1 Our Hypothesis of *MIR376B* regulated autophagy in HCC pathogenesis.

4.1 Outcomes of the thesis

Outcome I-Role of *MIR376B*-regulated autophagy attenuation in HCC proliferation

We have introduced that autophagy defects were associated with increased oxidative stress and lead to genomic instability in the context of HCC. In our experiments, we also showed a similar phenotype in breast cancer cell lines, which represent *MIR376B*-drive autophagy attenuation. Both the accumulation of ROS and genomic instability are quite crucial in hepatocyte transformation. Our results suggest that autophagy have a contribution to the late-stage disease where regulation restricts chronic damage.

Outcome II- Role of *MIR376B*-regulated autophagy attenuation in tumor proliferation

In our hand, we demonstrate that short term growth attenuated, whereas in the long term period, *MIR376B* clones taking advantage of the growth in comparison to control clones. Thus, our system suggest that chronic and acute autophagy deficiency may affect the cancer phenotypes particular in HCC.

Outcome III- Role of *MIR376B*-regulated autophagy attenuation in cell size control

Our MIRNA clones are susceptible to grow faster in the long term. However, we demonstrate that *MIR376B* clones also manifest cellular enlargement. Thus, according to our data, we proposed that autophagy-deficient cell-derived tumors may have bigger cells than control counterparts.

Outcome IV- Role of *MIR376B*-regulated autophagy attenuation in hepatocarcinogenesis

Lastly, *MIR376B* clones developed significantly bigger tumors in nude mice xenografts. Moreover, we also obtained data that *MIR376B* overexpression increase HCC cell migration and invasion. In addition, *MIR376B* expression level found to be high in almost 35 % of the total HCC patients and it is almost correlated with BECN1 level. Thus, our experimental data support various substantial evidence that *MIR376B*-involved in HCC progression.

4.2 Contributions to the literature

According to our findings, *MIR376B*-related therapeutics exert quite a significant potential, especially for HCC. It has a tremendous potential first as a diagnostic marker where we detected upregulation in 35 % of the HCC. Moreover, we declared many direct correlations in HCC progression, including growth, migration, invasion, and tumorigenesis. It is very promising in terms of its usage as a drug, especially for this fatal disease.

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APPENDIX A: PUBLICATIONS

PUBLICATION LIST,

^x Corresponding author. * First authors with equal contribution.

Original Research and Review Articles (Pressed)

- 1) Kocaturk N.M., **Akkoc Y.**, Kig C., Bayraktar O., Gozuacik D., Kutlu O^x, Autophagy as a molecular target for cancer treatment. *European Journal of Pharmaceutical Sciences*, 2019. **(Review; Citation Index-SCI, IF: 3,5)**
- 2) Zuvin, Merve; Kocak, Muhammed; Unal, Ozlem; **Akkoc, Yunus**; Kutlu, Ozlem; Yagci Acar, Havva; Gozuacik, Devrim; Kosar^x, Ali. Nanoparticle Based Induction Heating at Low Magnitudes of Magnetic Field Strengths for Breast Cancer Therapy. *Journal of Magnetism and Magnetic Materials*, 2019. **(Citation Index-SCI, IF: 3)**
- 3) **Akkoc Y.**, Lyubenova L., Grausgruber H., Janovská D., Yazıcı A., Çakmak I^x, Gozuacik D^x. Minor cereals harbor superior antioxidant effects on human epithelial cells compared to common wheat cultivars. *Journal of Cereal Science*, 2019. **(Citation Index-SCI, IF: 2,3)**
- 4) **Akkoc Y.** and Gozuacik D. Autophagy and liver cancer. *Turkish Journal of Gastroenterology*, 2018, 29:270-282. **(Review; Citation Index-SCI, IF: 0,9)**
- 5) Yar Y., Khodadust R., **Akkoc Y.**, Gozuacik D., Utkur M., Saritas U.E., Yagci Acar H^x. Development of Tailored SPION-PNIPAM Nanoparticles by ATRP for Dually Responsive Doxorubicin Delivery and MR Imaging. *Journal of Materials Chemistry B*, 2018, 6, 289-300. **(Citation Index-SCI, IF:4,5)**
- 6) Motezakker A., Sadaghiani A.K., **Akkoc Y.**, Parapari S.S, Gozuacik D., Kosar A. Surface modifications for phase change cooling applications via crenarchaeon *Sulfolobus solfataricus* P2 biocoatings. *Scientific Reports*, 2017, 7(1):17891. **(Citation Index-SCI, IF:4,8)**
- 7) **Gozuacik D^x**, **Akkoc Y.**, Ozturk DG, Kocak M., Autophagy regulating miRNAs and cancer. *Frontiers in Oncology*, 2017, April;7:65 **(Citation Index-SCI, IF: 4,3)**
- 8) Oznur Bayraktar, Ozlem Oral*, Nur Mehpare Kocaturk*, **Yunus Akkoc**, Karin Eberhart, Ali Kosar, Gozuacik D. IBMPFD disease-causing mutant VCP/p97 proteins are targets of autophagic-lysosomal degradation. *PLOS ONE* 11 (10), e0164864, 2016. **(Citation Index-SCI, IF: 3,5).**

- 9) Tekirdag, Kumsal Ayse; **Akkoc, Yunus** and Gozuacik, Devrim^x. MIR376 family in cancer. **Histology and Histopathology**, 2016, March 4:11752 (Review; Citation Index-SCI, IF: 2,1)
- 10) Oral, Ozlem; **Akkoc, Yunus**; Bayraktar, Oznur; Gozuacik, Devrim^x. Physiological and Pathological Significance of Molecular Cross-talk Between Autophagy and Apoptosis., **Histology and Histopathology**, 2016, May;31(5):479-98 (Review; Citation Index-SCI, IF: 2,1)

Book Chapters,

- 1) Gözüaçık D, Karakaş HE, **Akkoc Y**, Özata B. Otofaji ve kanser (Autophagy and cancer). **In the book: Kanser Moleküler Biyolojisi**. Yusuf Baran (Ed.).

Conference Published Abstracts,

- 1) **Akkoc Y.**, Yar Y., Khodadust R., Gozuacik D., Yagci Acar H.F. Development of Theranostic PNIPAM/ SPION Nanoparticles for Cancer Treatment. **Turkish Journal of Molecular Biology & Biotechnology**, 2017, 2(1), Supplement (1) page:84. ISSN 2149- 0627 (International).
- 2) **Akkoc Yunus**, Tekirdag, Ayse Kumsal, Ekici-Doğan Asiye Isin, Gözüacik Devrim. MIR376B in breast cancer. **Turkish Journal of Biochemistry**, 2016, 41(Supplement 4) page:135. e-ISSN 1303-829X p-ISSN 0250-4685 (International).
- 3) **Yunus Akkoc**, Kumsal Ayşe Tekirdağ, A. Işın Doğan-Ekici, Devrim Gözüaçık. MIR376 Family and Cancer. **Turkish Journal of Biochemistry**, 2016, 41(Special Issue 4) page:150. e-ISSN 1303-829X p-ISSN 0250-4685 (International).