Role of miRNA-126 in Hepatocellular Carcinoma and Cholangiocellular Carcinoma

By

Samar Abdullah Zailaie

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In

Molecular Pathology

Department of Laboratory Medicine and Pathology

University of Alberta

Abstract

Hepatocellular carcinoma (HCC) and Cholangiocellular Carcinoma (CCA) represent the most common malignant tumor of the liver accounting about 90 % of all liver malignancies. The overall prognosis of HCC and CCA is very poor due to the lack of effective treatment.

MicroRNAs (miRNAs) represent a small endogenous non-coding RNAs that play a significant role in the regulation of gene expression post-transcriptionally. Altered expression of miRNAs has been observed in many malignancies including liver cancer. However, the expression level of miR-126 in HCC and CCA and its role in hepatic-carcinogenesis remains unclear. This thesis aimed to study the expression level, localization and biological significance of miRNA-126 in HCC and CCA.

In an effort to distinguish the expression pattern of miR-126 in HCC and CCA tissues and cell lines, two expression analysis has been used: in situ hybridization (ISH) and quantitative real time polymers chain reaction (QRT-PCR). Our ISH analysis has shown a significant reduction in miR-126 level in HCC and CCA tissues relative to their corresponding normal tissues. Moreover, an intensive expression of miR-126 in normal hepatocyte, blood vessels and sinusoid cells has been observed. Our qRT-PCR data demonstrated a lower expression level of miR-126 in HCC and CCA cell lines relative to a normal kidney cell line. By using several gain of functions analysis, this study demonstrated the effect of miR-126 in HCC and CCA cell lines. The over-expression of miR-126 in HepG2 and HuccT1 has significantly inhibited cell proliferation and growth. On the other hand, our data has shown that miR-126 overexpression inhibited cell ability to migrate.

Taken together, this study indicted that miR-126 could play a critical role in hepatic carcinogenesis. Indeed, miR-126 may serve as a novel suppressive miRNA in liver cancer. Furthermore, miR-126 may serve as potential therapy, diagnostic and prognostic biomarker.

I dedicate this thesis to my lovely husband, Samer Frati, and my son Nawaf

Preface

This thesis is an original work by Samar Zailaie. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Proteomics of cholangiocellular carcinoma cell lines and role of miRNA" No. Pro00029122, 2012**.**

Acknowledgment

First and foremost, thank God for the wisdom that he has been given to me during my research project. Also, I owe my sincere appreciation to King Abdullah Bin Abdul-Aziz, may God be merciful to him, for giving me the opportunity to study abroad. All my gratitude to the Saudi Arabian Cultural Bureau in Canada for their great support during my study.

I want to thank my supervisors Dr. Consolato Sergi for his support, aid and dedicated commitment. All my thankfulness to my co-supervisor Dr.Judith Hugh and my committee member Dr.Fiona Bamforth for all their support, valuable advices and assistance in the completion of this study. I also would like Dr. Xing-Zhen Chen to serve as the external examiner and Dr.Monika Keelan to serve as exam chair.

I am also indebted to my colleagues Reem Abdulmajid, Yasser Abuetabh and Redha Albahrani for their steadfast encouragement on my research. I owe my honest acknowledge to Dr.Roger's Leng Lab for all their great advices and insight they have shared with me in my work. Furthermore, I am very appreciative to Kim Formenti for her technical help in my methods. I am deeply grateful to all my friends for sharing their truthful guidance.

Last but not least, I am deeply appreciative of my beloved husband Samer Frati, for his patience and encouragement. To my son, thank you for sharing me with my work, and for bringing a smile to my heart. I would like to express my deep sense of gratitude to my Mother for all her prayer that gave me the strength to pursue my studies, without her none of this would be accomplished. Many thanks to my father, sisters and brothers for their spiritual support.

List of Abbreviations

AFB1	Alpha-toxin B 1
AFP	Alpha-fetoprotein
AID	Activation-induced cytidine deaminase
Ago	Argonaute protein
AP	Alkaline phosphatase
ADAM9	ADAM metallopeptidase domain 9
Bcl-2	B-cell lymphoma 2
CD34	Hematopoietic progenitor cell antigen CD34
CDK	Cyclin-dependent Kinase
CT	Computerized axial tomography
C.sinensis	Clonorchis Sinensis
CCA	Cholangiocellular Carcinoma
CA19-9	Carbohydrate antigen 19-9
CEA	Carcinoembryonic antigen
C.elegans	Caenorhabditis elegans
CSD	N-terminal cold shock domain
CCHC-type	Two-zinc knuckle–type domain
CLL	Chronic Lymphocytic Leukemia
CDKs	Cyclin-dependent kinases
DCP	Des-Gamma-Carboxy Prothrombin
DGCR8	DiGeorge syndrome critical region 8
Ds-miRNA	MicroRNA duplex

DNMTs	DNA methyltransferases
EXP5	Exportin-5-dependent transports
EC	Endothelial cells
EGFL7	Epidermal Growth Factor Like Domain 7
ESCC	Esophageal squamous cell carcinoma
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal regulated kinase
FBS	Fetal Bovine Serum
FFPE	Formalin fixed paraffin embedded tissue
GPC3	Glypican-3
GOLPH2	Golgi phosphor- protein 2
GGT	Serum gamma glutamyl transferase
GTP	Guanosine-5'-triphosphate
GW182	Multiple glycine-tryptophan with molecular mass of 182kD
HCC	Hepatocellular Carcinoma
HBV	Hepatitis B virus
HCV	Hepatitis C virus
hAgo	Human Argonaute protein
IL-8	Interlukin-8
iNOS	Inducible Nitric oxide synthase
IL-6	Interleukin-6
ISH	In Situ Hybridization

LT	Liver transplantation
MRI	Magnetic resonance Imaging
Mcl-1	Myeloid cell leukemia-1
miRNA	MicroRNA
mRNA	Messenger RNA
mi-RISC	RNA-induced silencing complex
MCL-1	Myeloid cell leukemia-1
MTS	3-4,5-dimethylthiazol-2-yl-5 3carboxymethoxyphenyl-2-4-sulfophenyl-2H-tetrazolium
NF-KB	Nuclear Factor Kappa B
NO	Nitric oxide
NCI	NOD/SCID female mice
O.vierrini	Opisthorchis viverrini
PEI	Percutaneous ethanol injection
PSC	Primary sclerosing cholangitis
pri-miRNA	Primary micro-RNA
pre-miRNA	Precursor miRNA
PACT	Protein kinase R-activation protein
PIWI	P-element induced wimpy testis
PAZ	Piwi Argonaut Zwille domain
PDCD4	Programmed cell death 4
PI3K	Phosphatidylinositol 3-kinase signaling pathway
PIK3R2	Phosphatidylinositol 3-kinase regulatory subunit beta

15-PGDH	NAD-Linked 15-hydroxyprostaglandin dehydrogenase
RFA	Radiofrequency Ablation
RLC	RISC Loading Complex
RT-PCR	Quantitative real time polymerase chain reaction
RSK	Ribosomal S6 kinase
SIRT	Selective Internal Therapy
ssRNA	Single Strand RNA
TNF	Tumor Necrosis Factor
TGF-p	Transforming Growth Factor
TP53 or p53	Tumor suppressor 53
TRBP	TAR RNA binding protein
TP53INP1	Tumor Protein 53-Induced Nuclear Protein 1
TIMP3	Tissue inhibitor of metalloproteinase
3' UTRs	3' untranslated regions
VEGF	Vascular endothelial growth factor VEGF
ZnF	Zinc-finger domain

List of Tables

List of figures

Table of Contents

Chapter 1

Introduction

1 Malignant epithelial tumors of the Liver

Liver tumors are classified into two major categories: primary liver tumors and metastatic liver tumors. The primary tumor originates in the liver, while the metastatic tumor spreads to the liver from other organs of the body, accessing the liver through the portal vein or the hepatic artery. Primary liver tumors are further subdivided into benign or malignant; benign tumors are noncancerous and remain in the liver, and malignant tumors are cancerous and spread beyond the liver (1).

Most primary tumors are classified according to the liver cells they most resemble: hepatocytes, the bile duct epithelium, or the endothelium. Primary malignant epithelial tumors include hepatocellular carcinoma (HCC), hepatoblastoma, cholangiocarcinoma (CCA), and hepatic cystadenocarcinoma. This chapter discusses the two types of primary malignant epithelial tumors: HCC and CCA (1).

1.1 Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) constitutes a primary malignant tumor occurring in the hepatocytic cells account for 85-90% of all primary liver cancer (2). HCC currently ranks as the fifth most common type of cancer and the third most common cause of cancer-related mortality worldwide (2). According to the most recent statistics on the GLOBOCAN web site (2012), over 782,000 new cases have been diagnosed and a similar high number of deaths have also been reported.

HCC comprises a very poor overall prognosis, as approximately 80% of patients present with advanced metastatic tumors for which surgical resection lacks effectiveness. Some patients are diagnosed at an early stage of the tumor, which makes them eligible for curative treatment, including resections of the tumor by hepatectomy and liver transplantation. While approximately 30% of patients are eligible for these treatment options, the prognosis remains poor due to the

recurrence of HCC after surgery (3). On average, HCC patients survive only 7 months from the diagnosis period and approximately 10% of patients can live for 5 years following the surgery (2).

1.1.1 Epidemiology and risk factors

The incidence of hepatocellular carcinoma varies among different geographical regions. A high incidence of HCC occurs in developing countries, such as Southeast Asia and Sub-Saharan Africa, where chronic hepatitis B infection occurs in endemic proportions. In fact, 50% of all HCC cases have presented in China (4,5). Although HCC occurs less frequently in developed countries, its frequency has been increasing in developed countries, including the United States and Canada.

Several etiological factors fulfill a critical role in HCC pathogenesis. These factors include chronic viral hepatitis Type B and Type C infections (HBV, HCV), which include 80% of all HCC cases (6). In addition, liver cirrhosis represents another major risk factor associated with heptocarcinogenesis, as approximately 80-90% of HCC relates to cirrhosis (6). On the other hand, alcohol abuse has a significant involvement in the development of HCC. While the precise mechanisms involved in alcohol-induced hepatic carcinogenesis remain controversial, heavy alcohol consumption of more than 80g per day for at least 10 years and liver damage through oxidative stress are strongly associated with HCC (6). Additionally, the exposure to certain environmental toxins, especially Alphatoxin B1 (AFB1), also correlates with an increased risk of HCC pathogenesis (6). Many other possible risk factors enhance the likelihood of HCC, including the being male, smoking, diabetes, and obesity (6).

1.1.2 Pathogenesis

The pathogenesis of HCC entails a multifaceted mechanism involving several well-recognized risk factors that enhance the malignant transformation of liver hepatocytes. The

etiological factors discussed above represent jeopardy triggers to heptocarcinogenesis. Generally, HBV infection alone causes half of the HCC cases (7). While the molecular mechanism of HBV-induced tumorigenesis remains partially obscure, the majority of HBV-related HCC patients have developed cirrhosis of the liver upon diagnosis of HCC (8). Research suggests that chronic HBV infections induce inflammation of the hepatocytes as well as liver cell necrosis, subsequently increasing the synthesis of cytokine-induced fibrosis and eventually causing cirrhosis (8). This mechanism may result from the integration of HBV DNA into the cellular host genes of infected hepatocytes that regulate the growth of normal cells and thus lead to chromosomal deletions and genomic instability (8-10). On the other hand, studies have demonstrated that the viral transcription of the HBx protein can activate the secretion of cytokines, including nuclear factor kappa B (NF-KB), interlukin-8 (IL-8), tumor necrosis factor (TNF), and transforming growth factor (TGF-p) (11,12). All of these cellular genes are responsible for the growth and survival of cancer cells. In addition to the activation of cytokines by HBx, the inhibition of tumor suppressor 53 (TP53 or p53) by HBx can directly promote cell proliferation and inhibit normal cellular processes such as apoptosis (11,12). In contrast to HBV, HCV lacks the ability to integrate into a host genome such as HBV. However, HCV induces tumorigenesis by viral protein interactions, which leads to the inactivation of the cyclin-dependent kinase (CDK) inhibitor p21(13).

As previously mentioned, long-term alcohol consumption as well as chronic HBV/HCV causes chronic inflammation of the liver, increasing the secretion of fibers and collagen deposition in the liver. Eventually, these processes lead to cirrhosis, which constitutes a premalignant stage. Recent studies suggest that alcohol usage can directly induce hepatic carcinogenesis and increase the risk of HCC incidents in patients with viral hepatitis (14). Furthermore, dietary exposure to AFB1 is strongly linked to HCC. Research noted that HCC patients who were exposed to AFB1 acquired a mutation of tumor suppressor p53 at codon 249,

which caused the transversion of AGG^{Arg} to AGT^{Ser} (15).

1.1.3 Clinical, laboratory diagnosis and tumor markers

HCC patients are frequently diagnosed only when the tumor reaches its late stages due to the absence of pathognomonic symptoms; hence, HCC comprises is an asymptomatic disease in its early stage. Accordingly, the delay in HCC diagnosis diminishes the effectiveness of curative treatments, leading to a median survival rate less than one year after diagnosis. Because of tumor metastases and invasions, HCC usually entails a poor outcome. Patients with advanced HCC present with abdominal pain, hepatomegaly with a right upper mass, fever, weight loss, jaundice and anorexia (16).

The diagnosis of HCC involves the assistance of various laboratory tests and diagnostic imaging; so that, patients at a high risk of HCC can be engaged in a surveillance program with screening tests offered every 6-12 months (17). Patients with HBV, HCV, or alcoholic cirrhosis are considered as high-risk populations. Diagnostic imaging of the liver includes magnetic resonance imaging (MRI), computerized axial tomography (CT) scans, and ultrasonography.

The most frequently used laboratory test for HCC screening is the assessment of serum alpha-fetoprotein (AFP) levels. Despite the reputation of AFP as the most useful serum protein for HCC diagnosis, the sensitivity and specificity of remains low due to the possibility of detecting AFP in patients with chronic hepatitis and cirrhosis (17). In addition to AFP, other tumor markers include Glypican-3 (GPC3) and Golgi phosphoprotein 2 (GOLPH2) as well as several enzymes, such as serum gamma-glutamyl transferase (GGT) and Des-Gamma-Carboxy Prothrombin (DCP) (17,18).

1.1.4 Prognosis and potential challenges of HCC treatment

The treatment options for HCC vary based on the clinical findings, the time of diagnosis, and the stage of the tumor. Early detection of the tumor increases the likelihood that the tumor

can be treated. Unfortunately, only a small fraction of HCC patients are diagnosed early and can receive potential treatments. The treatment options for early HCC detection include surgical resection, liver transplantation, radioembolization, and percutaneous treatment (2,19,20). For patients presenting with advanced stage HCC, chemotherapy, namely Sorafenib, in combination with other treatments, such as radiofrequency ablation, may decrease the tumor and extend the patient's life (2,19). Sorafenib acts by blocking the neovascularization process and limiting the blood supply to the tumor cells (2)

The most effective treatment for HCC involves the surgical resection of the tumor; however, patients undergoing resection must meet strict criteria. For instance, patients must have normal liver function, possess a single nodule, and lack evidence of liver cirrhosis. (2). Liver transplantation (LT) also represents a potential curative treatment for HCC patients. Similar to surgical resection, patients must fulfill several criteria before physicians recommend LT. Candidates for LT typically have a cirrhotic liver containing one tumor lesion less than 5 cm or up to 3 lesions, each of which is 3 cm or smaller. Surgical resection and LT provide patients with five-year survival period: for surgical resection, 70-75% of patients experience a high risk of tumor recurrence, while for LT, only 15% of patients have a probability of tumor recurrence.(2,21).

In the 1980s, researchers established the benefit of percutaneous treatment of HCC. Specifically, two groups of investigators showed the ability of percutaneous ethanol injection (PEI) to suppress tumor growth (20). Several years later, PEI comprised a first-line treatment for early HCC tumors smaller than 3 cm in diameter, achieve a cure rate of 70-100%. Approximately half of patients with tumors between 3 cm and 5 cm demonstrated an effective response to PEI (18). Since that time, PEI has been replaced with radiofrequency ablation (RFA), another percutaneous technique, which provides early HCC patients with greater survival benefits in

comparison to PEI (2,18).

Radioembolization, also known as selective internal radiation therapy (SIRT), has recently undergone development as a technique for the treatment of intermediate-stage HCC. In SIRT, radioactive particles are delivered through the arteries that feed the tumor, subsequently causing apoptosis to the tumor cells (2).

1.2 Cholangiocellular carcinoma

Cholangiocellular carcinoma (CCA) is a primary liver cancer considered as a rare adenocarcinoma; however, the cancer ranks as the second common primary hepatobiliary malignancy after HCC (22). CCA arises from the epithelial cells of bile ducts; this cancer is classified according to the anatomical location of the tumor in the biliary tree: intrahepatic (ICC) and extrahepatic (ECC) (23). Extrahepatic CC further subdivides into distal extrahepatic and peripheral extrahepatic, also known as Klatskin tumors (22,24).

Similar to HCC, CCA is asymptomatic and patients present with an advanced stage, when therapeutic options are limited. At diagnosis, most patients have an untreatable tumor with a median survival rate of 12 months after diagnosis, and less than 5% of patients who undergo tumor resection survive up to 5 years. Overall, CCA entails a very poor outcome with a high mortality rate due to liver failure, biliary obstruction and persistent sepsis (23).

1.2.1 Epidemiology and risk factors of CCA

CCA represents the second most common primary liver cancer, the sixth most common cancer worldwide, and the third cause of cancer-related death(24). Over the last 30 years, the average age of CCA incidence occurs in the seventh decade with a 5:1 ratio of males to females(24,25). While CCA occurs most frequently in Asia, particularly Southeast Asia, the incidence of this cancer has been increasing in the US and Europe over the past four decades (24,26).

While the etiology of CCA remains unknown, many risk factors have been linked to CCA development. This include, Primary Sclerosing Cholangitis (PSC) that contributes to CAA within 2.5 years from its diagnosis (26). Currently, PSC is responsible for up to 15% of all CCA cases (26). Moreover, Kurathong S, *et al.* proposed the involvement of hepatobiliary flukes (*Opisthorchis viverrini and Clonorchis Sinensis*) in the pathogenesis of CCA; these flukes mainly occur from the consumption of undercooked or raw fish (27). The correlation between *O. viverrini* and the development of CCA has been approved in animal models (28). Furthermore, hepatolithiasis, which is defined as the presence of gallstones in the bile ducts, has been linked with the development of CCA (26,29). Additional risk factors for CCA include Caroli's syndrome, choledochal cysts, and congenital hepatic fibrosis (26).

1.2.2 Pathogenesis:

In the last decade, the incidence of CCA has increased; with a high mortality rate from intrahepatic CC. Several studies contributed to deciphering the molecular pathogenesis involved in the malignant transformation of cholangiocytes. These studies revealed the involvement of multiple etiological factors in cholangiocarcinogenesis with marked genetic and epigenetic alterations. However, research acknowledges that the chronic inflammation of the bile duct leads to cholangiocyte damage and subsequent obstruction of bile flow, thus leading to the development of CCA (30). One of the well-recognized risk factors contributing to the chronic inflammation of the biliary tree is PSC, a cholestatic liver disease characterized by the inflammatory destruction of the biliary tree (31). PSC causes a local increase of cytokine production, leading to prolonged stress in cholangiocytes, irreversible DNA damage, and the inhibition of the tumor suppressor gene (30,31). Recent studies revealed a high expression level of activation-induced cytidine deaminase (AID), the DNA/RNA-editing enzyme family, in PSC-related CC (32). The abnormal expression of AID has been associated with tumor suppressor

gene mutations such as p53 (32-34). On the other hand, a study investigating PSC-related CC showed an aberrant expression of the proto-oncogene k-ras (35) and inducible nitric oxide (NO) synthase (iNOS) (33). CCA patients infected with *O. viverrini* demonstrated a high expression level of cytokines, including the interleukin-1 receptor (IL-1) (36). Moreover, in vitro studies showed a correlation between bile duct acid accumulation and the development of CCA (37). Specifically, the accumulation of intrahepatic bile duct acid leads to the chronic inflammation of cholangiocytes, which subsequently increases the production of interleukin (IL)-6. IL-6 has proven to upregulate myeloid cell leukemia-1 (Mcl-1), a potent anti-apoptotic B-cell lymphoma (Bcl)-2 protein, which causes cellular resistance to apoptosis (30,37).

1.2.3 Clinical, laboratory diagnosis and tumor markers

Despite the availability of many diagnostic approaches, the diagnosis of cholangiocellular carcinoma remains difficult because most patients present with non-specific symptoms. This characteristic enhances the difficulty of diagnosing the disease in early-stage patients, with the exception of high-risk individuals enrolled in a screening program. Currently, the most effective method of CCA diagnosis involves the use of imaging techniques in combination with several laboratory tests. The two imagining techniques in CCA diagnosis include computed tomography (CT) and magnetic resonance imaging (MRI) (38). Among these methods, CT is most effective for assessing extrahepatic metastasis (39).

Serum levels of the tumor marker, carbohydrate antigen 19-9 (CA19-9), can detect CCA with 74% sensitivity and 82% specificity (40). CA19-9 occurs in high levels in patients with other gastrointestinal diseases, such choledocholithiasis (41). Since elevated levels of CA19-9 are correlated with metastatic CCA, its serum levels might be utilized as an indicator for tumor stages (42). In addition to CA19-9, carcinoembryonic antigen (CEA) has been elevated in CCA patients, demonstrating 52% sensitivity and 55% specificity (40).

1.2.4 Options and potential challenges of CCA treatment

CCA represents an aggressive tumor with a poor prognosis and a low survival rate. CCA patients live approximately 12 months or less without treatment (43). Definitive curative treatments have limited availability due to the difficulty of early diagnosis. In most cases, the resectability rate is very low due to the tumor's advanced or metastatic state (44). Currently, surgical resection and liver transplantation (LT) represent the only treatment options for early stage tumors (44). A complete tumor resection can be performed in 25–40% of CCA patients (45), with 20% of these patients having a three-year survival rate and 51% of patients having a one-year survival rate (43).

In the case of nonresectable tumors, LT comprises a preferable option for selective patients with tumor sizes of less than 3 cm and lacking evidence of extra or intra tumor extensions; however, even after LT, CCA patients still demonstrate a poor prognosis due to tumor reoccurrence (CCA (46,47). The survival rate of patients can be enhanced by the combination of tumor resection and neoadjuvant chemoradiation therapy, especially in comparison with tumor resection alone. (48).

1.3 miRNA

1.3.1 Overview

Since discovering that the lin-4 gene of *Caenorhabditis elegans* encodes small RNA complementarity to lin-14, researchers have suggested that the interaction between lin-4 and lin-14 fulfills a key role in the regulation of protein expression in *C. elegans* (49,50). Subsequent identification of the conserved let-7 gene among species including humans (51) has initiated a revolution in the scientific research to investigate a new class of small non-coding RNA, known as microRNAs (miRNA).

miRNA comprise a class of short (~22 nucleotides), non-coding RNA that functions as

post-transcriptional regulatory molecule in most eukaryotes, including humans, and provides a critical function in every aspect of biological processes, including proliferation, differentiation, developmental time, apoptosis, metabolism, and metastasis (51). Thus, the deregulation of miRNA has been highly associated with human diseases such as cancer (52).

The standard mechanism of action by which miRNA repress the translation of target proteins occurs through base pairings to the 3' untranslated regions (3'UTRs) of target messenger RNAs (mRNAs). This pairing mediates protein repression by mRNA cleavage or/and translational inhibition (52). The 3' UTRs of most mRNAs contain a conserved sequence that is highly complementary to a 2-8 bases of miRNA sequences; accordingly, these regions have been subsequently referred to as the 'seed regions' (53). However, mRNA cleavage requires a perfect base pairing with the guide miRNA, which does not usually occur in animals. Current research suggests that animal miRNA regulates gene expression post-transcriptionally by the process of translation inhibition (52). This mechanism, which involves the miRNA silencing gene expression, undergoes detailed discussion in Section 1.3.3.

1.3.2 miRNA biogenesis

1.3.2.1 General miRNA biogenesis aspect

The earliest steps of miRNA biosynthesis start in the nucleus, where the miRNA-genes are transcribed into primary miRNA (pri-miRNA), a long transcript with a hairpin structure, a terminal loop, and a flanking single strand RNA segment (52,54). An early study on randomly selected pri-miRNA showed evidence of miRNA gene transcription by RNA polymerase II (55), which initiates the transcription by binding to the promoter region of the miRNA gene. However, the miRNA-gene promoter lacks all elements required for transcription initiation (55), which complicates the identification of miRNA promoters. The majority of miRNA exist within the intrinsic and extrinsic regions of protein-coding genes; however, several studies have revealed

that some miRNA are located within the intergenic region (56-59)

Following transcription, pri-miRNA is processed through a sequence of endonucleolytic cleavage catalyzed by two RNA polymerase type III enzymes: Drosha and Dicer. In the nucleus, Drosha complexes with DiGeorge syndrome critical region 8 (DGCR8) to cleave the pri-miRNA into a ~ 60-100 nt hairpin structure precursor miRNA (pre-miRNA). Subsequently, pre-miRNA undergoes transport from the nucleus into the cytoplasm by exportin-5-dependent transports (EXP5) for additional cleavage by Dicer (52,60).

Dicer promotes the cleavage of pre-miRNA to generate a ~ 22 nt mature miRNA duplex (ds-miRNA) containing both guide strand and passenger strand miRNA. The miRNA duplex is then incorporate into an Argonaute protein to form a large protein complex termed the RNA-induced silencing complex (mi-RISC), which causes the degradation of the miRNA passenger strand and the unwinding of the miRNA duplex. Subsequently, the RISC complex guides miRNA to its target mRNA and represses the translation by base pairing to the 3'UTRs of its target (52). (Figure 1)

1.3.2.2 Nuclear processing of miRNA by Drosha

The stem-loop structure of the pri-miRNA transcript represents a distinctive characteristic that triggers Drosha to initiate the cleavage. Accordingly, Drosha acts on the base of the stem-loop to generate ~ 60 nt precursor miRNA (pre-miRNA) with around 2 nt overhanging at the 3'-end(49). An in vitro study indicated the necessary existence of a cofactor to increase the catalytic action of Drosha (61,62). This research found that the reconstructed Drosha fails to produce a mature miRNA, thus suggesting that Drosha requires an essential cofactor in order to generate a mature miRNA. For instance, DGCR8 is a cofactor that interacts with Drosha to form a large complex, known as the Drosha microprocessor complex. The main function of DGCR8 involves the recognition of the region between single stranded RNA and the stem-loop, which promotes

Drosha to cut ~11 bp away from the ssRNA-dsRNA junction (61).

The expression level of the Drosha/DGCR8 microprocessor represents a critical factor that regulates pre-miRNA production. Although the mechanism controlling the microprocessor expression remains unclear, recent studies suggest that the microprocessor's deregulation can alter miRNA production, which subsequently affects the function of cells. Muralidhar and his group have found that the expression level of Drosha increases in cervical cancer. These authors also found that Drosha is linked to the upregulation of miR-31 (63). On the other hand, *in vitro* processing of the pri-miRNA study has found that a minor change in DGCR8 expression inhibits the processing of pri-miRNA by Drosha. This finding suggests the tight regulation of the Drosha/DGCR8 microprocessor level, which is closely linked for the precise production of pri-miRNA(64).

1.3.2.3 Nuclear export of pre-miRNA to the cytoplasm

After nuclear processing mediated by the Drosha/DGCR8 complex, pre-miRNA is transported to the cytoplasm by Exportin 5 (EXP5) , RanGTP-dependent dsRNA-binding protein, for maturation. EXP5 recognizes pre-miRNA by its structure, thus indicating the significance of the steam loop for successfully binding to EXP5 (65).

The experimental knockdown of EXP5 causes a reduction in the overall level of mature miRNA (66). Interestingly, this study reveals the lack of accumulation of pre-miRNA in the nucleus, suggesting that EXP5 prevents nuclear digestion and facilitates the translocation of pre-miRNA into the cytoplasm (66). Pre-miRNA binds to EXP5 in conjunction with GTP, which is bound to the Ran cofactor following a release of pre-miRNA in the cytoplasm and a subsequent hydrolysis of GTP (54)

1.3.2.4 Cytoplasmic processing by Dicer and maturation

Following the translocation of pre-miRNA into the cytoplasm, another RNase type III

protein called Dicer cleaves pre-miRNA near the terminal loop to generate a ~22 nt mature miRNA duplex with 3' overhanging ends (49)

Similar to the case with Drosha, several studies have identified Dicer-associated proteins that act to enhance the stability and activity of this enzyme. The most abundant co-factor proteins found to interact with Dicer include the TAR RNA binding protein (TRBP) and the protein kinase R-activation protein (PACT) (67,68). The expression level of Dicer and its cofactor fulfill a critical role in miRNA biogenesis. A study conducted by Melo *et. al*. has demonstrated a key finding about miRNA biogenesis in human carcinoma. Their results suggest that the depletion of either TRBP or PACT affect the stability of Dicer, both of which are associated with a decreased level of miRNA (67). On the other hand, different expression levels of Dicer have been observed in several types of cancer; for instance, a decreased level of Dicer occurs in non-small cell-lung carcinoma and a high expression level exists in prostate cancer (49).

Cell lineage abnormal 28 (Lin28) is a conserved RNA-binding protein found in eukaryotes. This protein functions as a regulatory molecule in several cellular functions, including development, differentiation, metabolism, pluripotency and tumorigenesis. In recent years, lin28/lin28b has been found to modulate miRNA biogenesis by directly inhibiting pre-miRNA-Dicer processing(69). The negative regulation of miRNA biogenesis depends upon the domain structure of lin28/lin28b, which consists of three RNA-binding domains: an N-terminal cold shock domain (CSD) and two-zinc knuckle–type (CCHC-type) zinc-finger (ZnF) domains. The RNA-binding domains CSD and Znf have been implicated in miRNA processing (70). To date, only let-7 family members have been found to interact with lin-28(70). This relationship leads to the subsequent inhibition of Dicer action, revealing the post-transcriptional regulatory role of lin28 in Dicer activity (71).

1.3.2.5 Argonaute loading and RISC assembly

The miRNA duplex generated by Dicer becomes incorporated into the Argonaute protein (Ago) in order to form the RISC effector complex. Once loaded, the miRNA guiding strand remains in the Ago protein while the other strand of the duplex experiences degradation. Although the reason for selecting the guiding strand remains unclear, some studies investigating the miRNA duplex suggest the role of thermodynamic stability in strand selection(71). These studies found that the strand with an unstable base pair characteristic at the 5' end joins the Ago protein while the other strand with the more stable 5' end, becomes degraded (54).

Together, Ago and Dicer, in association with TRBP and/or PACT, form the RISC loading complex (RLC), which subsequently facilitates the formation of the RISC complex containing a single miRNA strand and guiding it to its target mRNA (49,54,71).

1.3.3 Mechanism of miRNA silencing gene expression

During the past decade, researchers suggested that miRNA play a key role in the posttranscriptional regulation of gene expression. Functional analysis of miRNA has revealed the precise ability of miRNA to recognize their target mRNA, bind to them by base pairing to the 3'UTR, and negatively regulating these targets. Currently, the two main mechanisms involved in miRNA silencing gene expression include mRNA cleavage and translation inhibition, also known as slicer-dependent and slicer-independent pathways (52). The miRNA-mRNA interaction strongly depends on the compatibility between the miRNA and the seed region of their target mRNA. The perfect match between miRNA-mRNA strands triggers mRNA degradation (Figure 1), while a lack of compatibility triggers translation repression (52).

miRNA mediate posttranscriptional gene silencing through the effector complex, RISCs, and its core component, Argonaute (Ago) proteins. Ago proteins comprise a class of highly conserved proteins in animals, plant, and fungi as well as in many unicellular and multicellular organisms. In humans, the Ago protein consists of four members: Ago1, Ago 2, Ago 3 and Ago

4. A structural analysis of Ago reveals the presence of two main domains: PIWI and PAZ (72,73). Each domain has its own unique characteristics that facilities the Ago mechanism of action. For instance, the PIWI domain contains an RNase H-like fold(73), which serves as the catalytic center of Ago responsible for targeted mRNA decay. In humans, only Ago2 (hAgo2) contains an active catalytic PIWI domain(62,74). Whereas the PIWI domain only occurs in Argonaute, the PAZ domain also exists in Dicer enzymes(75). Several studies suggest that the PAZ domain possesses the ability to interact with the 3′ end of pre-miRNA. In conjunction, the PAZ domain in Dicer and Argonaute proteins fulfill a key role in identifying the terminal structures of pre-miRNA and aid in its processing (75,76).

As previously mentioned, a perfect match between miRNA and its target mRNA promotes the endonucleolytic cleavage by Ago2. Although the majority of animal miRNA can promote the translation inhibition of their target (52), mRNA decays can also occur. The mechanisms by which miRNA mediates mRNA degradation remain only partially understood and controversial. However, studies targeting the Argonaute protein, especially Ago1, have demonstrated the importance of GW182 family proteins, which are required for the miRNA-mediated gene silencing via mRNA cleavage. These studies conclude that GW182 interacts with Ago1 and enhances decay via deadenylation and decapping with DCP1 and DCP2 (77,78).

In vitro experimental studies have revealed evidence of translation repression by miRNA in the two stages of protein synthesis: initiation and elongation. The implications of these findings have proposed several mechanisms by which miRNA mediates translation inhibition, including cap recognition and suppression, elongation blocking, deadenylation of targets mRNA by Ago, and 60S repression subunit joining(77,79,80).

1.3.4 The involvement of miRNA in cancer

The discovery of *Lin-4* miRNA in *Caenorhabditis elegans* has prompted investigations to

study the non-protein-coding sequences in DNA, which initially lacked recognized biological functions and were simply considered as 'junk DNA'. Shortly after the revelation of *lin-4* miRNA, this 'junk DNA' attracted scientific research attention to study the role of miRNA. Seven years later, a second miRNA, known as let-7, was discovered in *C.elegans*, where let-7 controls larval developmental time (51). In humans, let-7 demonstrates variable expression levels among different tissues, such as the heart, brain, and colon (51).

In 2002, the first correlation between miRNA and cancer was reported when Calin and his group investigated frequent chromosomal deletion in chronic lymphocytic leukemia (CLL) and two miRNA: miR-15 and miR-16 (81). These authors revealed a frequent deletion of chromosome 13q14 in the majority of CLL cases. In addition, they demonstrated that miR-15 and miR-16 are located within the deleted region. (51,82). A few years later, the miR-17-92 cluster was identified as a potential oncogene that controls the expression of c-Myc in a B-cell lymphoma mouse model(83,84). This cluster located in chromosome 13q31.3, a region that amplifies in several types of lymphomas(85,86). MiR-17-92 cluster demonstrates overexpression in colon cancer(87)and lung cancer(88). These findings suggest that miRNA genes are located in fragile sites of the genome that are usually deleted or amplified in human cancer(81).

Global analysis of miRNA in cancer tissue has shown a deregulation in total miRNA expression level when compared to the corresponding normal tissues. For instance, microarray and northern blot analyses reveal that several miRNA in breast cancer tissues are significantly deregulated, including miR-145, miR-125b, and miR-155(89). In colorectal neoplasia, miR-143 and miR-145 have demonstrated downregulation in comparison to normal mucosa specimens (90). Moreover, bead-based flow cytometric analyses of 217 miRNA expressions in 334 human samples have revealed that, in comparison to normal tissues, 90% of miRNA are downregulated in leukemias and many solid cancers (91). Conversely, large-scale analyses of miRNA profiling

in 540 samples from six solid tumors including breast, colon, stomach, prostate, pancreas, and lung showed 26 overexpressed miRNA and 17 downregulated miRNA (92). Specifically, miR-21 demonstrated overexpression in all of the six tumors; miR-218-2 is downregulated in stomach, colon, prostate, and pancreas cancers but not in lung and breast carcinomas(92).

1.3.4.1 Potential role of miRNA as tumor suppressors

The profiling of miR-15 and miR-16 in CLL comprised one of the first observations of miRNA involvement in cancer progression. A few years later, B cell lymphoma 2 (BCL-2) was identified as a main target of miR-15 and miR-16. Cimmino and his group have shown that these two miRNA were significantly downregulated in 26 CLL samples, which contributed to CLL tumorigenesis by upregulating Bcl2, an anti-apoptotic gene(93). Moreover, these authors also demonstrated that the overexpression of miR-15 and miR-16 downregulates BCL-2 and induces the apoptosis of B cells in CLL. Thus, the role of these miRNA in negatively regulating the BCL-2 gene indicated their tumor-suppressive properties.

Another group of miRNA that display tumor-suppressive properties include the let-7 family. As previously mentioned, this group of miRNA was originally identified as controlling larval developmental time in *C.elegans*(51). In 2004, Takamizawa *et al.* performed *in vitro* and *in vivo* studies to display a remarkably low expression of let-7 in human lung cancer (94). Furthermore, the overexpression of let-7 in human lung cancer cells inhibits cancer cell growth *in vitro*. Overall, these finding indicate the critical role of let-7 in tumorigenesis. Hence, these early observations of tumor suppressive properties in miR-15, miR-16, and let-7 provided a conclusion to scientific research that miRNA with reduced expression may act as a tumor suppressor gene by inhibiting oncogenes, anti-apoptotic genes, and genes that regulate cell proliferation and differentiation (51). Examples of other tumor suppressor miRNA are listed in Table 1.

1.3.4.2 Potential role of miRNA as oncogenes

In addition to tumor suppression, miRNA also contain well-established oncogenic properties. Oncogenic miRNA are overexpressed in cancer and a play a key role in the inhibition of tumor suppressor genes as well as apoptotic genes and regulator genes involved in cell proliferation and differentiation (51). The miRNA-17~92 cluster is a well-established oncomir known as on OncoMiR-1. In 2005, He *et al.* first reported the oncogenic aspect of miRNA-17~92(83). These authors found that the overexpression of miRNA-17~92 in c-Myc transgenic mice directly enhanced the formation of lymphomas(83). Although the exact mechanism of how miRNA-17~92 induces tumorigenesis in mice models remains unclear, one hypothesis maintains that it may result from c-Myc overexpression. (83).

Another example of an oncomir is miR-155, which has been commonly overexpressed in solid tumors, such as lungs (95), breasts(89), and pancreas (96) as well as human lymphomas (97). Studies have reported that miR-155 might act as an oncogene in pancreatic cancer through the inhibition of Tumor Protein 53-Induced Nuclear Protein 1 (TP53INP1) (96). Examples of other oncogenic miRNA are listed in Table 2.

1.3.5 Involvement of miRNA in hepatobiliary carcinogenesis

The frequent deregulation of miRNA has fulfilled a critical function in human cancer. For example, profiling studies conducted on hepatobiliary cancer have demonstrated signature-deregulated miRNA that participate significantly in hepatobiliary carcinogenesis. These miRNA control liver cancer development and participate in all tumor stages, including cell proliferation, metastasis and apoptosis. Table 3 summarizes some of the newly profiled deregulated miRNA in liver cancer, including HCC and CCA.

1.3.5.1 miRNA in HCC

In the past few decades, many studies have investigated the involvement of miRNA in

hepatocellular carcinoma progression, and emerging evidence has shown the effect of deregulated miRNA in HCC cell proliferation and cell resistance to apoptosis. Moreover, certain miRNA have been found to participate in tumor behavior, tumor stage, prognosis, and metastasis potential. The cloning and sequencing of miRNA in mouse models has resulted in the assignment of tissue-specific miRNA. Since 2002, miR-122 has been described as a hepatic-specific miRNA that has not been detected in another tissue(98).

Several studies have demonstrated that miRNA can affect cell proliferation by targeting genes involved in cell cycle control. For instance, cyclin-dependent kinases (CDKs) comprise well-known proteins from the kinase family that regulate the cell cycle. A recent study on the role of miRNA-200a in HCC progression demonstrated that the deregulation of miR-200a in HCC cell lines induce tumorigenesis by targeting CDK6 (99). Also, investigations have illustrated that CDK6 constitutes a direct target for miRNA-124(100). In addition to the regulation of cell cycle genes by miRNA, apoptotic genes comprise a direct target for miRNA. For example, one *in vitro* study has revealed a strong association between miRNA-122 and Bcl-w as a main target. In this study, the induced expression of miR-122 downregulated Bcl-w, suggesting that miR-122 may induce tumor cell apoptosis (101). IL-6 and MCL-1 also represent other targets for miRNA, including miR-125b and miR-26a(102,103).

The expression of miRNA in HCC also correlates with the clinical features of HCC patients. Specifically, the expression levels of miR-20, miR-92, miR-18 were significantly elevated in poorly differentiated HCC samples but remained constant in well-differentiated HCC samples (104,105).

1.3.5.2 miRNA in CCA

The involvement of MiRNA in CCA pathogenesis has not been studied as extensively as that in HCC. Consequently, the exact mechanism of miRNA-induced CCA tumorigenesis

remains controversial. However, investigations have reported several deregulated miRNA in CCA and their possible targets.

miRNA-21 represents one of the most studied miRNA in CCA. Kawahigashi *et al.* reported that among 28 miRNA included in their study, miR-21 expressed most frequently in CCA cell lines. In fact, miR-21 was also expressed in the normal intrahepatic biliary epithelial cell line, suggested the novelty of miR-21 in biliary epithelium (106). One recent study reported that the NAD-Linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH), the COX-2/PGE2 signaling pathway key enzymes, constitute a direct target for miR-21(107). PDCD4(108)and TIMP3(109) were also identified as potential targets for miR-21. Moreover, *in vitro* studies of miRNA-200b/c and miRNA-31 in CCA cell lines showed that these miRNA inhibited cell migration and cell proliferation by targeting ROCK2(110) and RASA1(111)respectively.

1.4 MiR-126

Expression analyses of miRNA have confirmed the existence of certain miRNA in a particular organ or to a specific cell linage. Additionally, recent studies on Dicer-miRNA interaction in endothelial cells (EC) have revealed that this relationship has a significant impact on EC functions (112,113). For example, an in vitro study on Dicer-allele hypomorphic mice has shown that the Dicer-miRNA interaction significantly impedes the formation of blood vessels in embryos and yolk sacs(114) Based on these findings, Dicer and miRNA may combine to perform a possible role in angiogenesis.

In 2008, an investigation on the expression of miRNA identified miRNA-126 as an endothelial-specific miRNA located within the 7th intron of the Epidermal Growth Factor Like Domain 7 (EGFL7), which resides in human chromosome 9 (115,116). Moreover, miR-126 demonstrates a high expression pattern in hematopoietic progenitor cells and highly vascularized tissue, including the liver(115). Moreover, the role of miR-126 has been recognized in several

diseases, such as atherosclerosis (117,118)and it also fulfills a significant role in pre-eclampsia pregnancy disorder (119).

1.4.1 miRNA 126 in cancers

The expression patterns and implications of miR-126 in several diseases have provided new insight for studying its role in cancer. The downregulation of miR-126 has been observed in different types of primary tumors, including breast, lung, pancreas, and gastrointestinal cancers. In breast cancer, miR-126 expression generally decreased in MDA-MB-231 cancer cell lines; the induction of its expression suppresses the ability of MDA-MB-231 to metastasize(120). Moreover, the *in vivo* delivery of miR-126 in NOD/SCID female mice (NCI) has significantly decreased the overall tumor size(120). These findings indicate that miR-126 contains the ability to suppress breast cancer growth and metastasis by inhibiting cancer cell proliferation. In lung cancer, expression profiling has revealed a low level of miR-126 in cancer tissue relative to non-cancerous control samples(121,122). Furthermore, miR-126 restoration in lung cancer cells has reduced the expression of vascular endothelial growth factor (VEGF) and inhibited cell proliferation(123). The delivery of miR-126 via lentivirus into nude mice successfully impeded the growth of A549 lung cancer cells *in vivo* (123). However, VEGF has been identified as a main target for miR-126 in many other types of cancers (115). The loss of miR-126 has been reported in many gastrointestinal cancers, including gastric cancer(124,125)and colon cancer(126-128). Interestingly, SOX2, CXCR4, and genes involved in the phosphatidylinositol 3-kinase signaling pathway (PI3K) have been identified as a novel target for miR-126 in gastric and colon cancer (125,126,128). The low expression of miR-126 has also been detected in pancreatic cancer (129).

1.4.2 miR-126 in liver cancer

Based on the literature and available data for miR-126, its expression pattern generally exhibits downregulation in most cancers, including liver cancer. To our knowledge, only one study on CCA tissue was reported (130). McNally *et al.* have demonstrated a low expression level of miR-126 in 32 restricted CCAs relative to adjacent normal bile duct epithelia. These authors also demonstrated a correlation between miR-126 and the overall survival rate; however, low expression was associated with improved median survival rates(130).

Studies have focused more strongly on miR-126 in HCC than in CCA. In 2012, Novellino *et al*. proposed that miR-126 tends to be low in HBV-related HCC(131). These authors showed that miR-126 expression, along with other miRNA, was significantly associated with immune-precipitated HBsAg particles from the sera of 11 HBV carriers, suggesting that miR-126 may act as a prognostic marker for HBV-related HCC(131). Moreover, investigators have reported that miR-126 expression levels were lower in tumor tissues of patients with recurrent HCC following liver transplants when compared to non-recurrent patients(132). In the same study, the authors also found that the restoration of miR-126 expression in HCC cell lines inhibit cancer cell migration, invasion, growth, and proliferation. Similarly, the overexpression of miR-126 possessed the ability to suppress HCC metastases *in vivo* (132). In addition, another research reported a decreased level of miR-126 in HCC recurrence tissue after LT (133). In contrast, qRT-PCR analysis on HCC tissue and cell lines has revealed a lower expression level of-miR-126 in comparison with normal liver tissue and cell lines (134). The same investigators have performed an *in vitro* gain of function analysis, showing a reduction in the ability of cells to invade and migrate. These results indicate that miR-126 possesses an anti-metastasis and anti-angiogenesis effect in HCC cancer cells by targeting LRP6 and PIK3R2(134). Interestingly, the same study performed an *in vivo* analysis using nude mice; the findings demonstrated the suppressive

properties of miR-126 in tumor proliferation and angiogenesis(134). Another study of HCC tissue has revealed a lower expression pattern of miR-126*, a miR-126 complement, in alcoholic-related HCC when compared to other types of HCC(135).

1.5 Rationale of this study, hypothesis and thesis objectives

An increasing number of liver cancer studies have aimed to identify unique diagnostic markers and therapeutic approaches by understanding liver cancer pathogenesis. The discovery of miRNA and the subsequent experiments of their biological functions *in vivo* and *in vitro* have provided significant insight into carcinogenic processes and tumor behavior. Specifically, the altered expression of miRNA in cancer tissues relative to non-cancerous tissues implies the role of miRNA in cancer development as novel oncogenes and tumor suppressors. MiR-126 has been investigated in many human cancers, and researchers have elucidated its functions as a tumor suppressor gene. To the best of our knowledge, existing studies have not provided insight about miR-126 in liver cancer, including HCC and CCA.

Therefore, this thesis examines the hypothesis that miR-126 fulfills the role of a tumor suppressor gene in liver cancer, especially CCA and HCC.

The **primary objective** of this thesis aims to investigate the expression level of miR-126 in HCC and CCA by using cell lines and formalin-fixed paraffin-embedding tissue as study models. Since miR-126 tends to be downregulated in most cancer types, the expression level of miR-126 in our study models was confirmed using in situ hybridization analysis (ISH) and quantitative real time polymerase chain reaction (RT-PCR). Upon the confirmation of miR-126 expression levels, the **second objective** aimed to use gain of functional analysis to study the biological effects of miR-126 in cancer cell proliferation, migration, and growth.

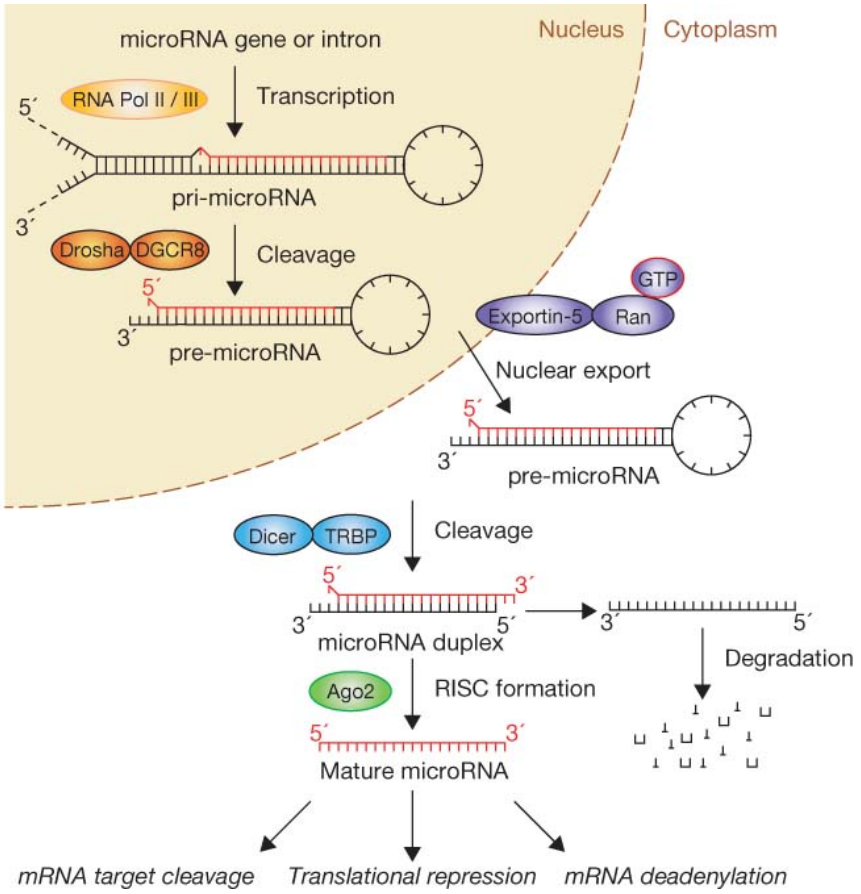


Figure 1.1 . miRNA biogenesis (71). In the nucleus, polymerase II or III transcript miRNA gene into primary miRNA transcript (pri-miRNA) which is then cleavage by the microprocessor complex Drosha–DGCR8. The pre-miRNA is then exported from the nucleus to the cytoplasm by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its 22-nucleotide mature miRNA duplex. The guiding strand of the miRNA duplex is then loaded with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it directs RISC to there target mRNAs through ad dampen their expression by mRNA degradation, translational repression or deadenylation. Reprinted with permission from the publisher.

Table 1: Examples of miRNA with tumor suppressive aspects

miRNA	Cancer	Target Gene	Reference
miR-18a	Colorectal Cancer	CDC42	(141)
miR-30a	Chronic Myeloid Leukemia	BCR-ABL	(142)
miR-127	Breast Cancer	Tudor-SN	(143)
miR-29	B Cell Chronic Lymphocytic Leukemia	TCL1	(144)
miR-181a	B Cell Chronic Lymphocytic Leukemia	TCL1	(144)

Table 2: Examples of miRNA with tumor oncogenic aspects

miRNA	Cancer	Target Gene	Reference
miR-222	Oral Squamous Cell Carcinoma	PUMA	(136)
miR-199a	Gastric Cancer	ZHX1	(137)
miR-135b	Osteosarcoma	FOXO1	(138)
miR-106a	Pancreatic Cancer	TIMP-2	(139)
miR-106a	Gastric Cancer	TIMP-2	(140)

Table 3: Examples of deregulated miRNA in Liver Cancer

miRNA	Liver Cancer	Expression	Proposed Function	Target	Reference
miR-29	CCA	Downregulated	Tumor Suppressor	c-Myc	(145)
miR-127	HCC	Downregulated	Tumor Suppressor	MMP13	(146)
miR-138	CCA	Downregulated	Tumor Suppressor	RhoC	(147)
miR-141	HCC	Downregulated	Tumor Suppressor	HNF-3β	(148)
miR-181a	HCC	Downregulated	Tumor Suppressor	c-Met	(149)
miR-198	HCC	Downregulated	Tumor Suppressor	c-Met	(150)
miR-204	CCA	Downregulated	Tumor Suppressor	Slug	(151)
miR-214	CCA	Downregulated	Tumor Suppressor	Twist	(152)
miR-362	HCC	Upregulated	Oncogene	CYLD	(153)
miR-433	HCC	Downregulated	Tumor Suppressor	PAK4	(154)
miR-486	HCC	Downregulated	Tumor Suppressor	p85α	(155)
miR-605	CCA	Downregulated	Tumor Suppressor	PSMD10	(156)

Chapter 2

Materials and Methods

2.1 Cell lines:

The previously published cholangiocarcinoma cell line, HuCCT1, was purchased from the cell culture bank of the Japan Health Sciences Foundation. Alternatively, the hepatocellular carcinoma (HCC) cell line, HepG2, was obtained from the American Type Culture Collection, (ATCC, Rockville, MD). The human embryonic kidney 293 cells, HEK293T, were obtained from Dr. Judith Hugh's Lab in the Katz Building at the University of Alberta.

All cells were cultured in their appropriate media. Specifically, HuCCT1 was cultured in a RPMI 1680 medium, purchased from Invitrogen Canada Inc. in Burlington, ON, Canada. This was supplemented with 10% Fetal Bovine Serum (FBS), obtained from PAA Laboratories Inc. in Etobicoke, ON, Canada, and with 1ml gentamicin. HepG2 was cultured in Eagle's Minimum Essential Medium (EMEM), purchased from ATCC in Rockville, MD, and supplemented with 10% FBS and 1ml gentamicin.

The cell line HEK293T was cultured in Dulbecco's Modified Eagle Medium (DMEM) and purchased from Invitrogen Canada Inc. in Burlington, ON, Canada. This specimen was supplemented with 10% FBS and 5 ml penicillin – streptomycin. All cells underwent incubation in a 5 % CO2 chamber at 37◦C and were grown as a monolayer.

2.2 Tissue samples:

Hepatocellular and cholangiocellular carcinoma tissues were obtained from the archives of the Department of Laboratory Medicine and Pathology at the University of Alberta. All tissue samples were fixed in 4% buffered formaldehyde and processed regularly. Paraffin tissue blocks were sectioned at six μm in an RNA-free environment and used for the *in situ hybridization method.*

2.3 RNA extraction, cDNA synthesis, and RT-PCR:

The total RNA was extracted from the aforementioned cell lines by using mirVana™

miRNA Isolation Kit, purchased from Ambion in Burlington, ON and applied according to the manufacturer's instructions. The quality and quantity of the RNA samples were assessed by the Agilent 2100 Bioanalyzer, located in the Department of Agriculture, Food and Nutritional Science at the University of Alberta, and the NanoDrop 1000 and Spectrophotometer, obtained from Thermo Scientific in Wilmington, USA. First, 10 ng of the total RNA underwent reverse transcription (RT) at a final reaction volume of 15 ul using the TaqMan® MicroRNA Reverse Transcription Kit, purchased from Applied Biosystems in CA, USA and applied according to the manufacturer's instructions. The cycling conditions for the RT reaction involved the following steps: 1) hold at 16 °C for 30 min, 2) hold at 42 °C for 30 min, and 3) hold for 5 min at 85 °C followed by a holding step at 4 °C for ∞. The expression level of mature miR-126 was measured by a real time PCR reaction (qRT-PCR) according to the TaqMan® MicroRNA assays protocol under the following cycling conditions: hold at 95 °C for 10 min followed by 40 cycles of denaturing at 95°C for 15 s and an annealing or final extension at 60°C for 30 s. All quantitation procedures were normalized using human U6 small nuclear RNA (U6 snRNA), obtained from Applied Biosystems, by the $2^{-\Delta ct}$ method. Theoretically, miR-126 expression level becomes downregulated in cancer cell lines when the relative expression ratio is less than one. The primers used in RT and qRT-PCR reactions for miRNA-126 (hsa-miR-126-3p, UCGUACCGUGAGUAAUAAUGCG) and U6 snRNA were purchased from Applied Biosystems in CA, USA. Both reactions were conducted using the StepOnePlus RT-PCR machine, obtained from Applied Biosystems.

2.4 Transient transfection of miRNA-126 mimic and negative control:

Chemically modified double-stranded RNAs that mimic miRNA-126 and mirVana™ miRNA Negative Control #1 were purchased from Ambion. Each day, the HuCCT1 and HepG2 cells were trypsinised, counted, and seeded in six well plates at a density of 200,000 cells/well.

This procedure was performed prior to the transfections to ensure 80% confluence at the time of transfection. Subsequently, the transfection of miR-126 mimic and negative control occurred using Lipofectamine® RNAiMAX Transfection Reagent, purchased from Invitrogen and applied according to the manufacturer's instructions, at a final concentration of 10nM. After 48-post transfections, qRT-PCR was used to confirm the transfection (Figure 5). The transfected cells were then used for subsequent experiments.

2.5 Cell proliferation assay:

To detect the changes in cell viability, an MTS assay was performed using CellTiter 96® AQueous One Solution Cell Proliferation Assay, obtained from Promega in Madison, USA and applied according to the manufacturer's instructions. 24 hours after transfection, the HepG2 and HuCCT1 cells were trypsinised, counted, and seeded in a 96 well plate at a density of 5 X 10^4 cells/well and grown for up to 3 days. The wells containing only media were used as a negative control. Every 24 hours, 20 ul of MTS reagent was added to all wells and the solution incubated for 4 h. Subsequently, the number of viable cells was assessed by measuring the absorbance at 490 nm using the SpecraMax M3 Microplate Reader, purchased from Molecular Devices in CA, USA. The experiment was conducted a total of three times.

2.6 Colony formation assay:

A colony formation assay was used to monitor cell growth. Two cells, HepG2 and HuccT1, previously transfected with either miR-126 mimic or negative control, were trypsinised 24 h after transfection. All cells were counted and seeded in six well plates at a density of 1000 cells per well and incubated with their common media for up to two weeks. Specifically, HuCCT1 underwent incubation for 10 days while HepG2 was incubated for 14 days. The colonies that appeared were fixed with 4% formaldehyde and stained with 0.1% crystal violet.

All colonies, with the exception of small colonies, were counted by the naked eye. This experiment was repeated three times.

2.7 Wound healing assay:

To detect changes in the ability of tumor cells to migrate, a wound-healing assay was performed. Specifically, 24 hours after transfection, HepG2 and HuCCT1 cells were trypsinised, counted, and seeded in six well plates and cultured overnight in their common media to ensure 90% confluency. The next day, the monolayer of the cells were wounded by a 200µl pipet tip. Then, cells were rinsed twice with 1x PBS to remove unattached cells, and the common media replaced. HuCCT1 cells were photographed at 0 and 12 h using an inverted digital microscope obtained from Zeiss microscope at the Department of Oncology of the University of Alberta in Edmonton, Canada. HepG2 cells were photographed at 0 and 48 h using the same microscope. The gap size between the wound edges was measured using TScratch software (157). Subsequently, the percentage of the open image areas at the end-time point in comparison to the 0 hour open area was measured by the following formula: [open wound area through time] = 100 x [open image area end-point] / [open image area 0h] .The experiment was conducted a total of three times.

2.8 In situ hybridization:

In situ hybridization was performed in FFPE tissue sections using the miRCURY microRNA ISH Optimization Kit # 5, obtained from Exiqon in Vedbaek, Denmark. This kit contains a Double-DIG-labeled LNA™ miR-126 probe, a scrambled negative control probe, and a U6 small nuclear RNA positive control probe. The negative control probe contained no complementary sequences among human and mouse genomes. All buffers or reagents used in the experiment were prepared as described in the kit's protocol. Sections were cut at 6 µm in an RNA-free environment at Pathology Core Translational Research Facility, located in The Li Ka

Shing Building at the University of Alberta, Edmonton, AB. The day prior to the experiment, all slides were baked at 60 °C for 2 hours in order to melt the paraffin and subsequently stored at 4°C overnight. The next day, slides were placed in xylene to remove the paraffin, and in ethanol to dehydrate. The xylene was applied three times for 5 minutes each, while a series of graded ethanol was applied in the following manner: 100% strength for 5 minutes, 96% strength for 5 minutes, and 70% strength for 5 minutes. These procedures were followed by a washing step in 1xPBS for 5 min. The Proteinase-K treatment was performed at a concentration of 15 ug/ml for 10 min at 37 ° in the Dako hybridizer, obtained from ThermoBrite StatSpin in Iris, USA. Next, a probe hybridization step was conducted for 60 min at 52 °C. The probe concentrations were 1 nM for the LNA U6 snRNA probe and 60nM for the Double-DIG-labeled LNA™ miR-126 probe / scrambled probe. Stringent washing steps were subsequently performed with SSC buffers at a hybridization temperature of 52°C. Subsequently, slides were incubated with an antibody blocking solution for 15 min at room temperature and then subjected to an incubation with an anti-DIG-regent having a concentration of 1:500 for 60 min at room temperature. In order to avoid tissue dehydration, the incubation occurred in a humidifying chamber. Slides were then incubated with NBT/BCIP alkaline phosphatase (AP) substrate, obtained at Roche in Mannheim, Germany, for 2 h at 30°C in the Dako hybridizer. Finally, slides were incubated with KTBT buffer (AP stop solution) twice for five minutes in order to stop the reaction. They were then counter stained with Nuclear Fast Red, obtained from Vector Laboratories in Burlingame, CA, USA, and mounted with Eukitt mounting medium, purchased from Sigma-Aldrich Co. LLC in Oakville, Ontario. All stained sections were visualized by using light microscopy with blue staining indicating of a positive signal.

2.9 Statistical analysis:

The Student t-test for two independent groups determined the statistical significance using IBM SPSS Software Version 22. P-values of less than 0.05 were considered significantly different.

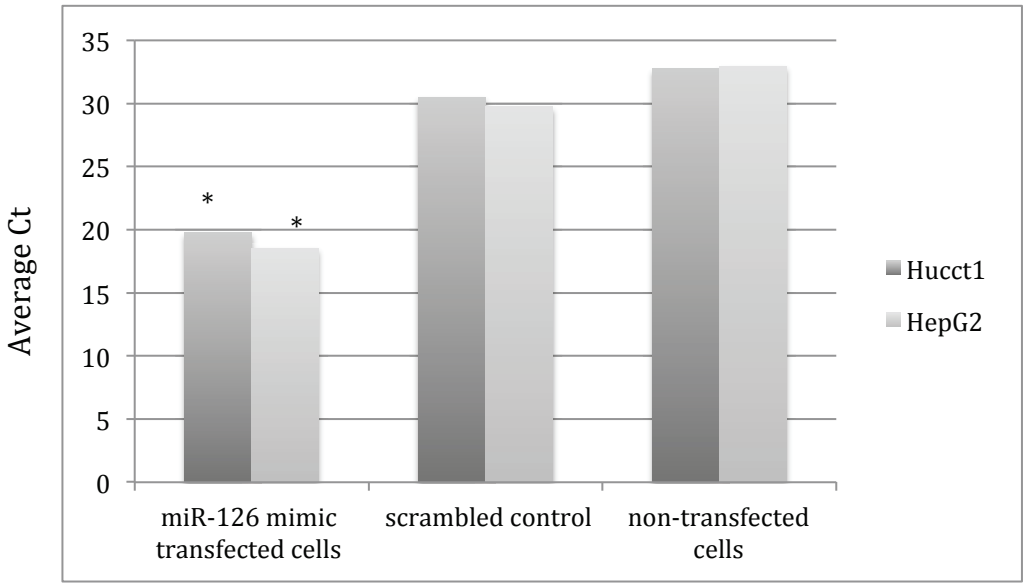


Figure 2.1: QRT-PCR for transfected cells. HepG2 and HuCCT1 cells were transfected with Lipofectamine® RNAiMAX at a final volume of 10nM. A Ct value of less than 20 was considered as a successful transfection. $P < 0.007$ (t.test)

Chapter 3

Results

3.1 The miRNA-126 expression pattern is lower in tumor cells relative to human embryonic kidney cells:

The role of miRNA-126 was investigated in the previously published hepatocellular (HepG2) and cholangiocellular carcinoma (HuCCT1) cell lines. First, the expression pattern of miR-126 in tumor cells underwent comparison to that in Human Embryonic Kidney 293T cells. The findings showed that the expression level of mature miR-126 is significantly lower in HepG2 and HuCCT1 cells relative to HEK293T. These results are demonstrated in Figure 3.1, ($P \leq 0.05$).

3.2 The miR-126 expression is high in normal hepatocytes and blood vessels in situ:

To visualize the expression pattern of miR-126, HCC and CCA tissues were used as a study model. In order to confirm the specificity of hybridization probes for our target, miR-126, the probes underwent adjustment for all tissues with positive U6snRNA probes and negative scrambled miRNA probes. The positive staining in nuclei occurs in Figure 3.2 while Figure 3.3 displays the negative scrambled probes without labeling.

The results demonstrate that miR-126 is highly expressed in normal hepatocytes of HCC tissues (Figure 3.4A and B) and CCA tissues (Figure 3.5A and B). However, miR-126 fails to show high expression at the tumor cells of the tissues (Figures 3.4C and 3.5C), especially in comparison to the negative and positive controls (Figures 3.4D and 3.5D). On the other hand, miR-126 showed a strong staining signal in the endothelial cells of blood vessels in both tissues (Figures 3.4 A and B and 3.5A and B). Interestingly, a positive signal of miR-126 has been observed in the sinusoid cells of HCC tissues (Figure 3.6).

3.3 The overexpression of miR-126 in tumor cells suppresses proliferation in HepG2 and HuCCT1 cells:

The two cell lines, HepG2 and HuCCT1, transfected by miR-126 mimic and scrambled shRNA, were subjected to a MTS assay to determine the change in cell proliferation. MTS data

revealed that the miR-126-transfected cells have a lower proliferation rate in comparison with those in the negative control group. A significant difference in the proliferation of HepG2 cells (Figure 3.7) was observed after 72h ($P<0.05$) and 96 h ($P<0.05$) post transfection. Similarly, HuCCT1 (Figure 3.8) showed significant differences after 48 h ($P<0.05$) , 72 h ($P<0.05$) , and 96 h ($P<0.05$) post transfection.

3.4 The overexpression of miR-126 in tumor cells suppresses growth in HepG2 and HuCCT1 cells:

To investigate the biological affect of miR-126 in cell growth, colony formation assay was used. Figures 3.9 and 3.10 reveal that miR-126 transfected cells significantly suppressed HepG2 and HuCCT1 cell growth relative to the negative control group. HepG2 and a significant HuCCT1 demonstrated a significant difference of $P<0.05$.

3.5 Effects of miR-126 mimics on regulation of HepG2 and HuCCT1 cells wound healing ability:

HepG2 and HuCCT1 cells transfected with miR-126 mimic and/or negative control were subjected to a wound-healing assay in order to investigate the biological effect of miR-126 in cell migration. As seen in Figures 3.11 and 3.12, the migration ability of HepG2 and HuCCT1 was significantly decreased when compared to that of the negative control. Specifically, HepG2 displayed a significant difference of $P<0.05$ at 0-48 h while HuCCT1 showed significant difference of $P<0.05$ at 0-12 hours.

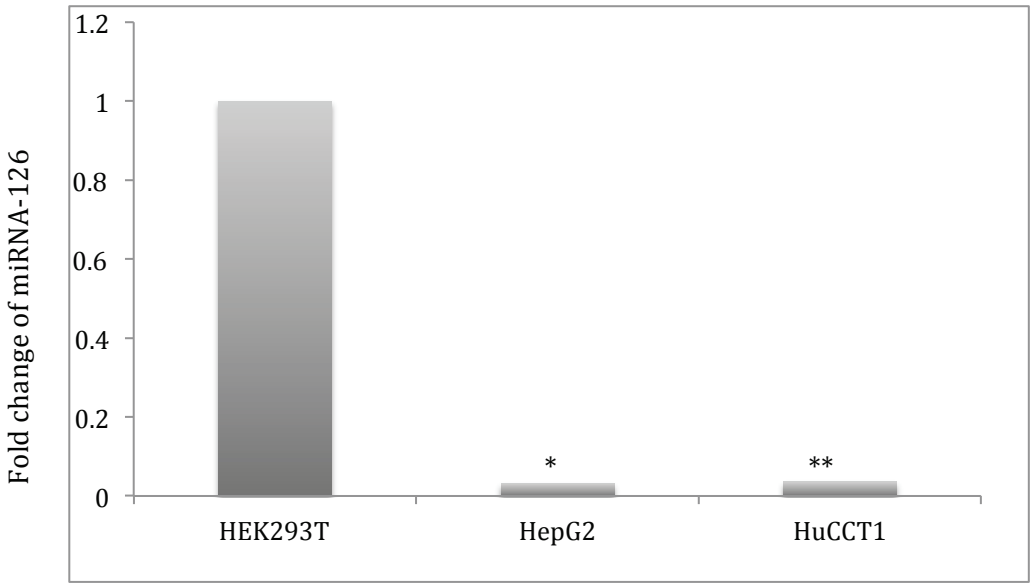


Figure 3.1: Results of qRT-PCR measuring miR-126 expression in HepG2 and HuCCT1 cells. MiR-126 showed a relatively lower expression in HepG2 and HuCCT1 cells in comparison to HEK293T. *, ** $P < 0.05$

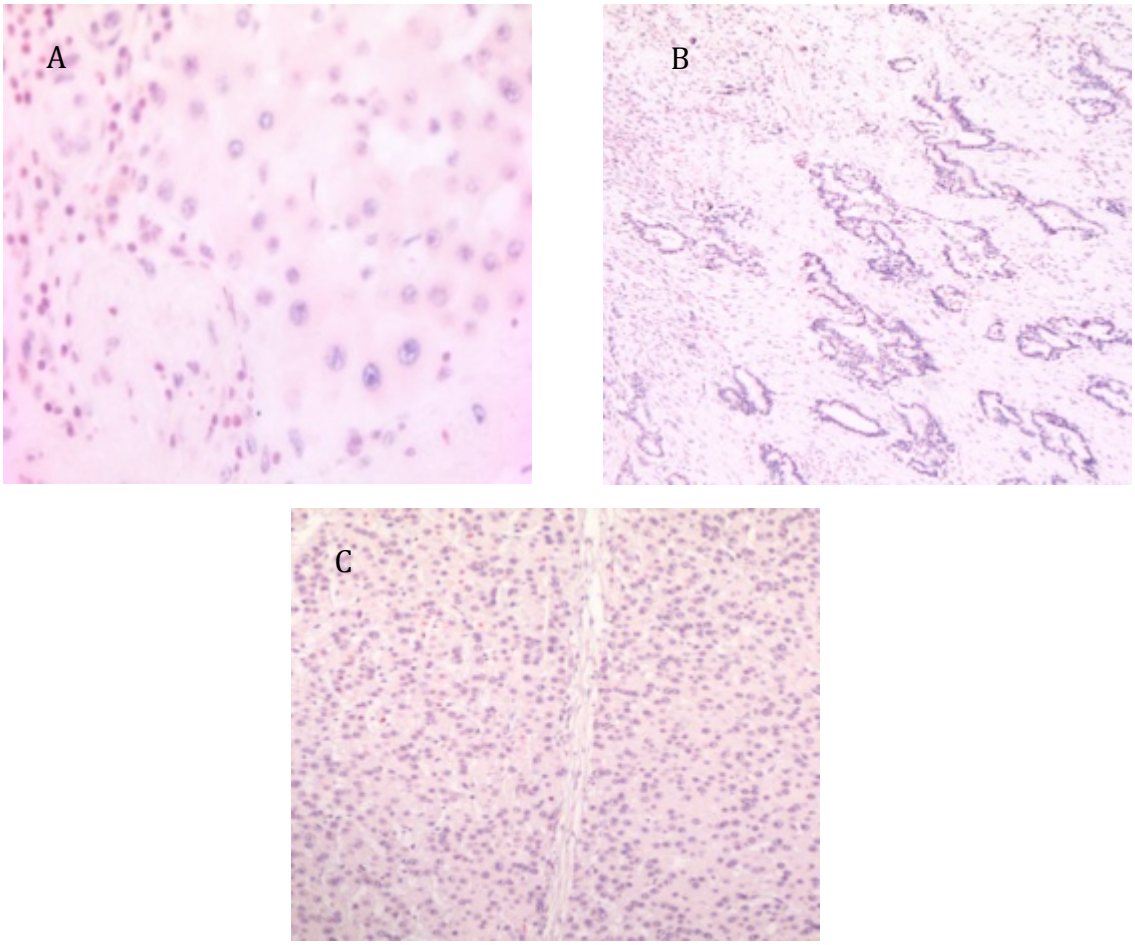


Figure 3.2: HCC and CCA FFPE tissue sections hybridized with U6 snRNA probe as a positive control. The blue signal indicates a positive signal **(A, B)** CCA FFPE tissue sections showed a strong nuclear positive hybridization signal for U6 (400x 100X respectively) **(C)** HCC FFPE tissue section showed a strong nuclear positive hybridization signal for U6 (100x).

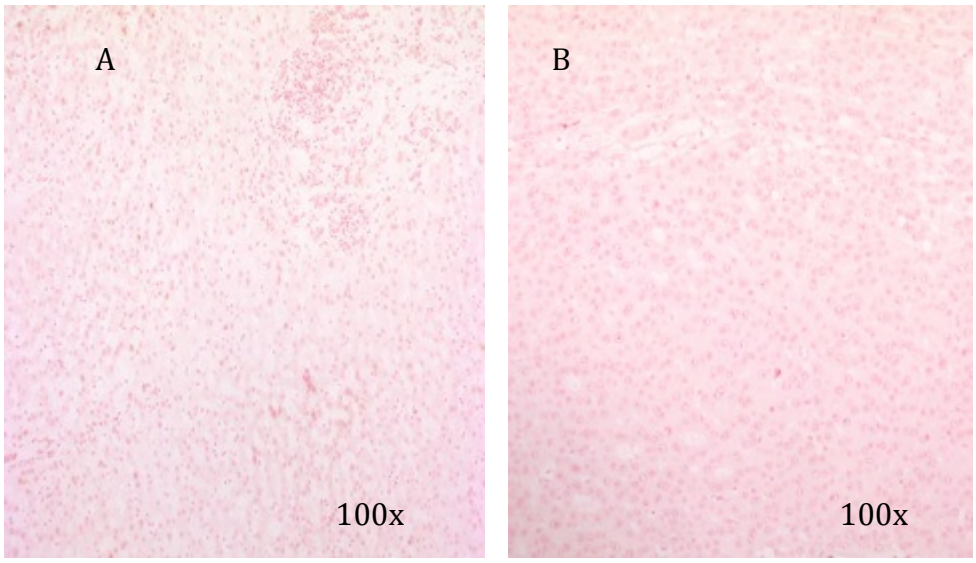


Figure 3.3 : HCC **(A)** and CCA **(B)** FFPE tissue sections hybridized with scrambled miRNA probes used as a negative control. (Magnification 100x)

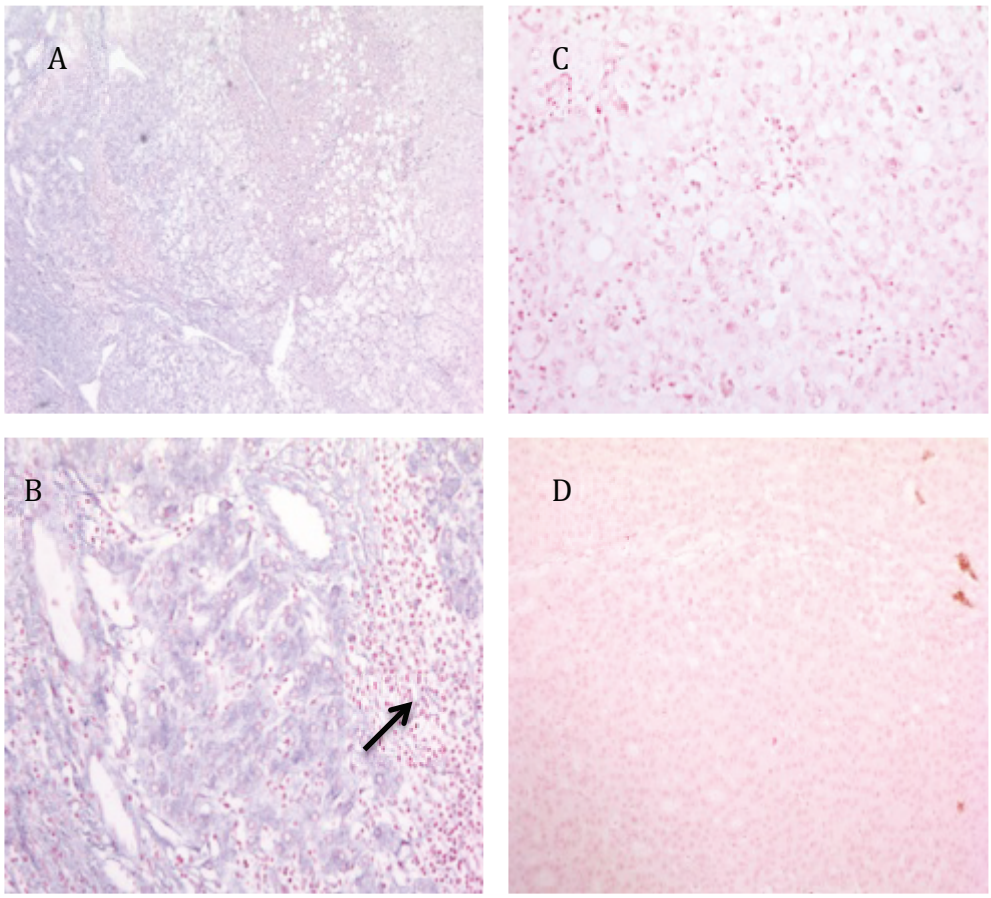


Figure 3.4: miR-126 expression in HCC tissue sections. The blue signal represents a positive labeling. **(A and B)** images represent extensive and high expression (blue) of miRNA-126 in normal hepatocytes and in blood vessels endothelium. MiR-126 expression is mainly distributed in the cytoplasm (magnification 50x). Arrow is pointed to tumor cells where no noticeable miR-126 expression (positive signals) was detected. **(C)** Image represent a lack of miR-126 expression in HCC tissue section hybridized with miR-126 probe (no labeling) (magnification 200x) **(D)** HCC tissue section hybridized with a scramble probe used as a negative control (100x).

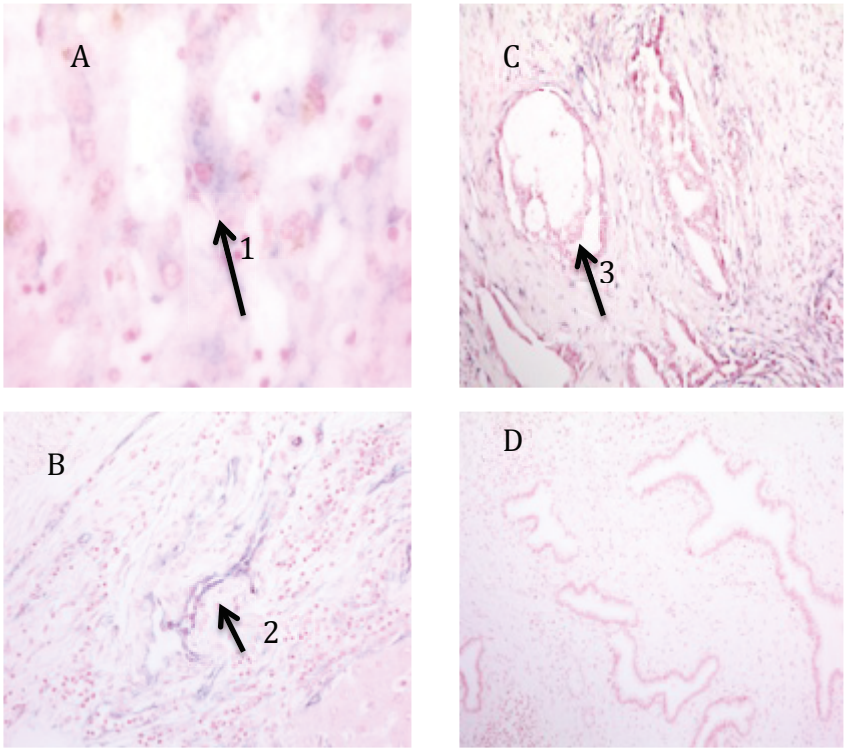


Figure 3.5: miR-126 expression in CCA tissue sections. The blue signal indicates positive labeling. **(A, B)** images represent an extensive and high expression (blue) of miRNA-126 in normal hepatocytes and blood vessels endothelium (magnification 400x, 200x respectively). Arrow **1** is pointed to a normal hepatocyte with extensively MiR-126 expression, which is mainly distributed in the cytoplasm. Arrow **2** is pointed to blood vessels with widespread miR-126 expression **(C)** Negative miR-126 expression in tumor cells (100x). Arrow **3** is pointed to tumor cells where no noticeable miR-126 expression was detected **(D)** Scrambled negative control probes (50x)

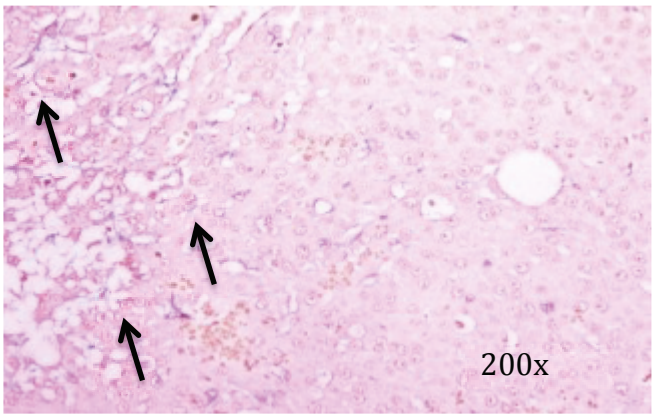


Figure 3.6: miR-126 expression in sinusoid cells of HCC tissue section. Image represents HCC tissue section hybridized with miR-126 LNA probe. Arrow is pointed to sinusoid cells with widespread miR-126 expression (blue signal).

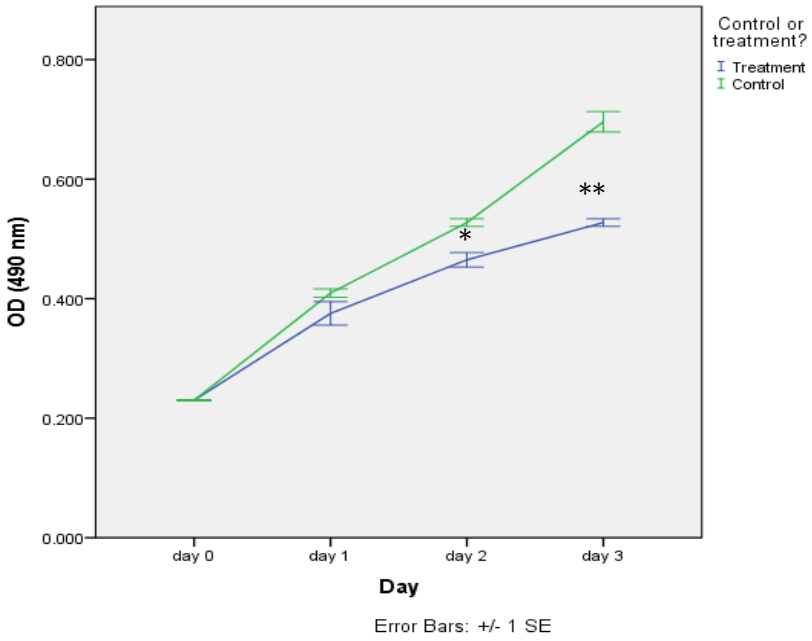


Figure 3.7: MTS data analysis for HepG2.The change in cell viability was measured by reading the optical density (OD) at 490nm. As seen in the graph, miR-12 over-expression inhibits the proliferation of HepG2 cells in comparison to NC. *'** P <0.05

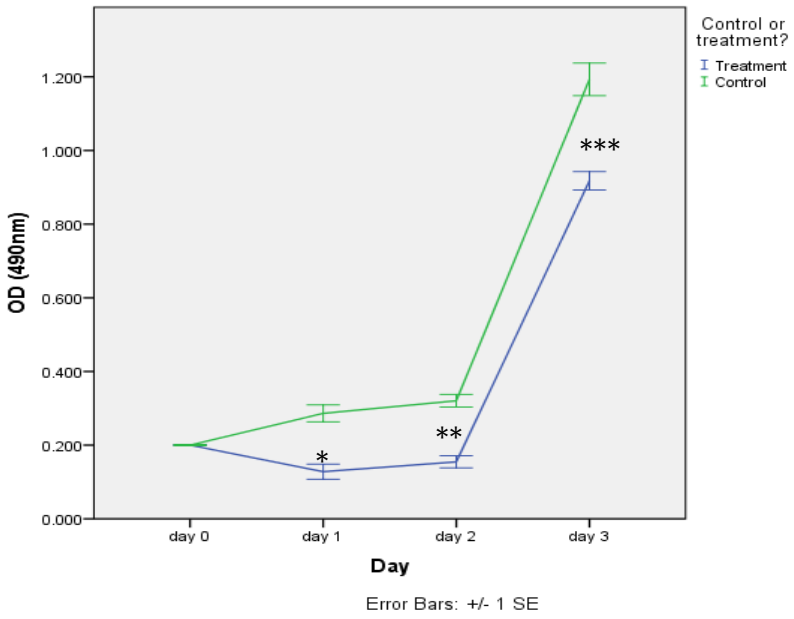
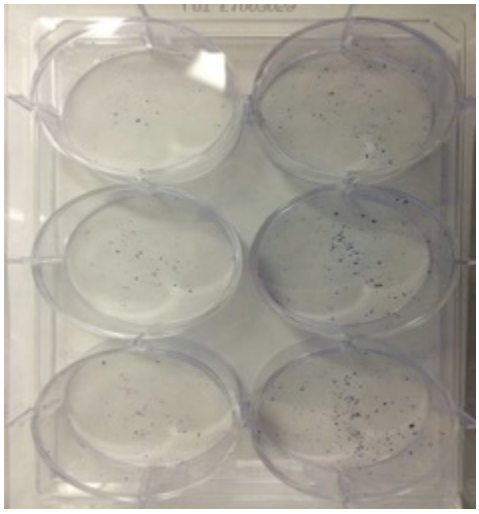


Figure 3.8: MTS data analysis for HuCCT1.The change in cell viability was measured by reading the optical density (OD) at 490nm. As seen in the graph, miR-126 over-expression inhibits the proliferation of HuccT1 cells in comparison to NC *'**'*** P <0.05



MiR-126 NC

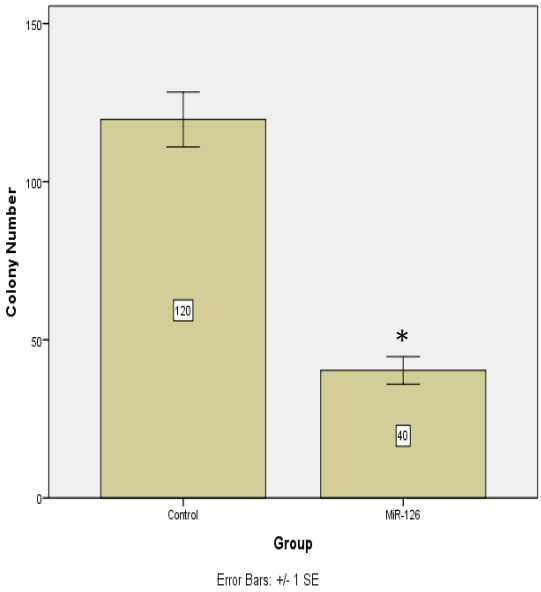
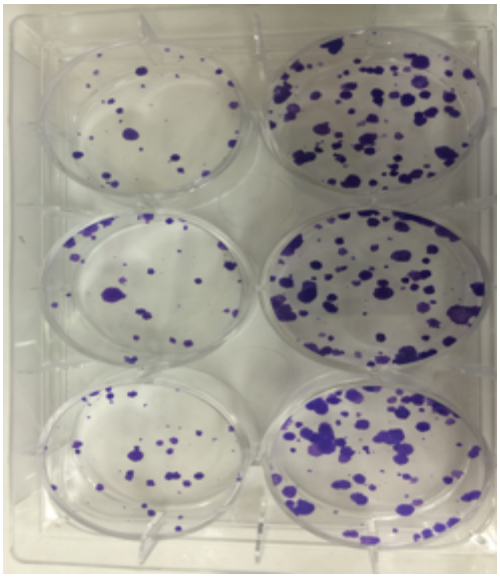


Figure 3.9: Colony formation assay for HepG2. MiR-126 transfected cells show lower colony numbers in comparison to the negative control (NC). *P<0.05



MiR-126 NC

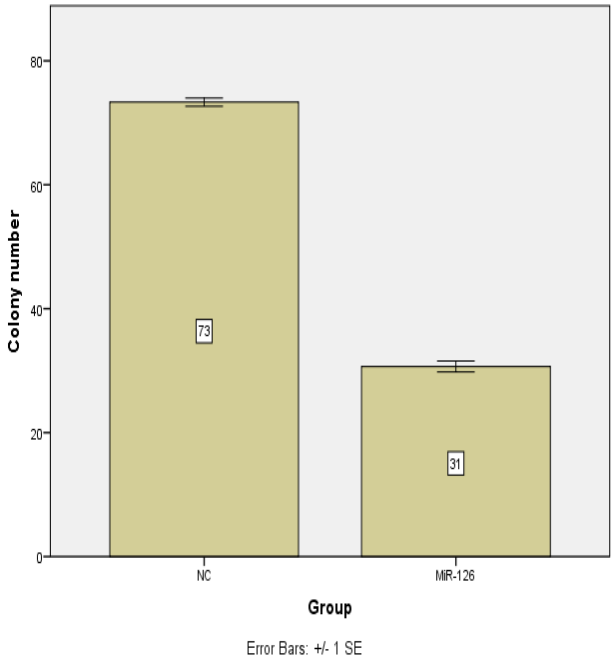


Figure 3.10: Colony formation assay for HuCCT1. MiR-126 transfected cells show lower colony numbers in comparison to the negative control (NC). *P<0.05

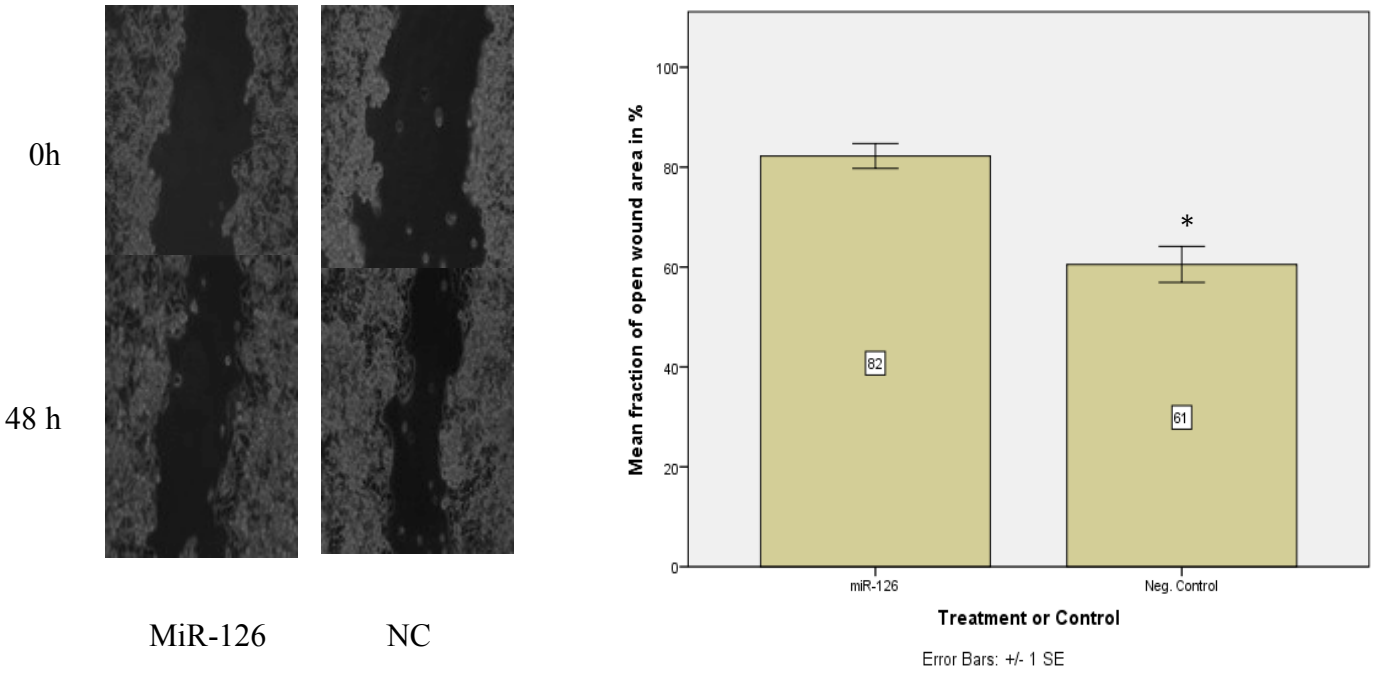


Figure 3.11: Wound-healing assay was performed to evaluate the effect of miR-126 overexpression on HepG2 migration ability. The over expression of miR-126 significantly impeded the HepG2 cells migratory ability. **(A)** 48 h post transfection, cells transfected with miR-126 mimic closed the wound more slowly than NC. **(B)** The graph shows the fraction of open wound areas in the form of a percentage. * $P<0.05$

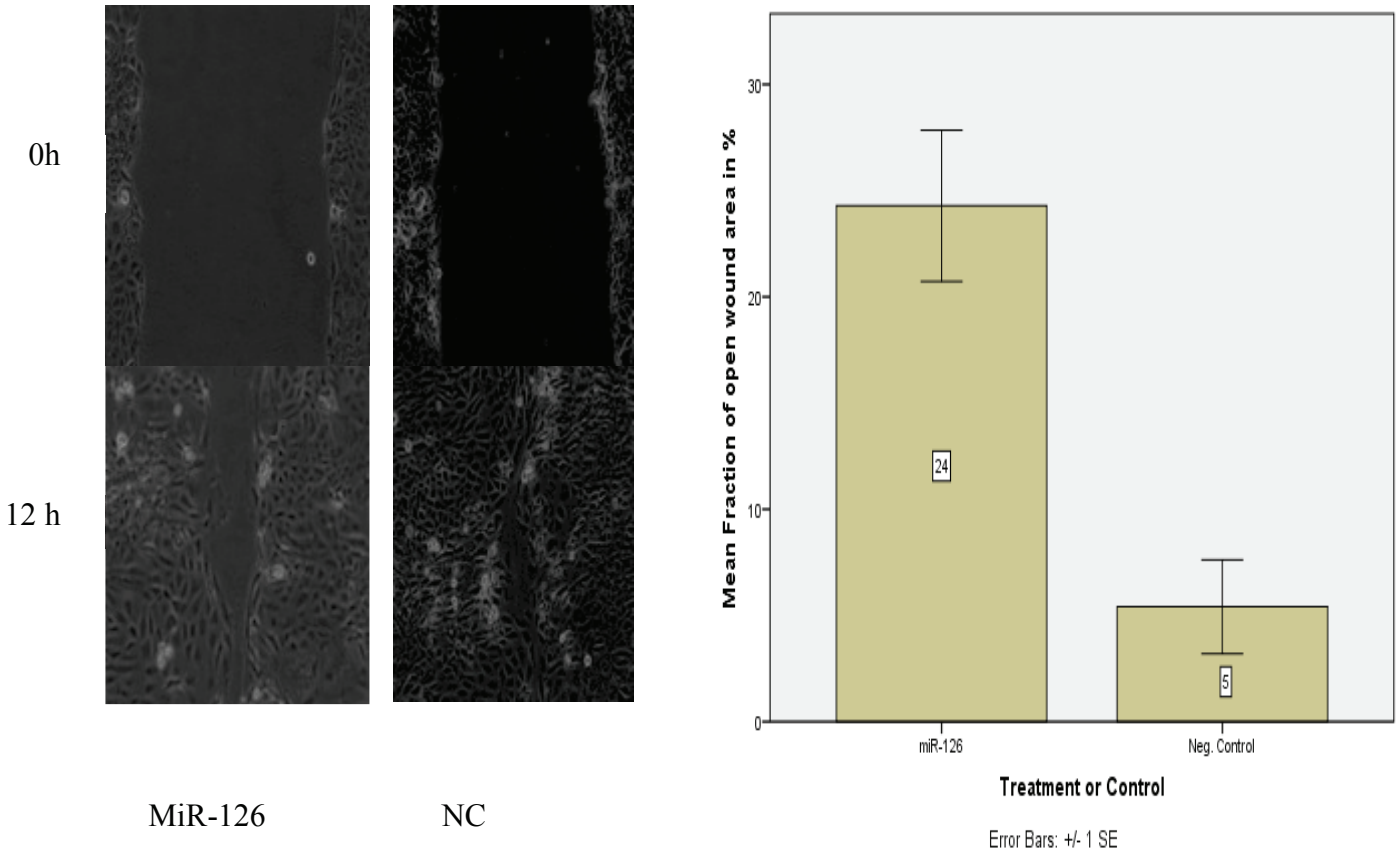


Figure 3.12: Wound-healing assay was performed to evaluate the effect of miR-126 overexpression on HuccT1 migration ability. The over expression of miR-126 significantly impeded the HuccT1 cells migratory ability. **(A)** 48 h post transfection, cells transfected with miR-126 mimic closed the wound more slowly than NC. **(B)** The graph shows the fraction of open wound areas in the form of a percentage. * $P<0.05$

Chapter 4
Discussion

4.1 General Discussion:

The aggressive nature of liver cancer emphasizes the importance of studying this type of carcinoma because of its high worldwide mortality rate. The most challenging aspect of liver cancer involves the wide variation in its etiological factors and its poor prognosis. The most common primary liver cancer is hepatocellular carcinoma (HCC) with cholangiocellular carcinoma (CCA) being the second most common primary liver malignancy. The diagnosis and treatment of HCC and CCA is difficult due to the absence of specific symptoms, earning the name "silent killers". Several studies have investigated the molecular aspects (e.g. miRNA) of liver malignancy in order to improve its diagnosis and treatment. In recent years, miRNA have been found to be involved in cancer progression and behavior; in particular, some miRNA have been highlighted as an organ-specific or cell type-specific miRNA. Lately, the endothelial specific miRNA, miR-126, has drawn research attention as a tumor suppressor gene that plays a vital role in cancer development. To date, few studies have focused on miR-126 in liver cancer, and a paucity of information exists concerning its role in HCC and CCA. In the present study I investigate the miR-126 expression in HCC and CCA tissues and/or cell lines. Moreover, an *in vitro* gain of function analysis has been applied in order to understand the biological effect of miR-126 in liver cancer progression.

4.1.1 Analysis of miRNA-126 expression pattern in HCC and CCA cell lines and tissue:

The expression pattern of miR-126 tends to be down regulated in several types of cancer, including gastric(122,124), colon (126-128), pancreas(129), lung(123), and breast (120). Moreover, the down-regulation of miR-126 in HCC tissue and cell lines has been previously reported (131-134). Accordingly, the present study demonstrates a low expression of miR-126 in previously published HCC and CCA cell lines, HepG2 and HuCCT1 respectively. These cell

lines underwent comparison to normal Human Embryonic Kidney cells (HEK293T) by qRT-PCR analysis. Specifically, the cell line HEK293T was selected due to the limitation of using normal liver cells.

In situ hybridization represents one of the most effective methods for analyzing the spatial expression of miRNA in tissue sections. The present study has determined the expression and localization of miR-126 in formalin-fixed HCC and CCA tissue sections by exploiting the locked nucleic acid (LNA) probe. As shown in the results, a strong signal of miR-126 occurred in normal hepatocytes as well as in the endothelial cells of blood vessels and capillaries. On the other hand, a weak or absent signal was observed in the tumor cells of HCC and CCA tissues, especially in comparison to the negative control. This finding indicates the loss of miR-126 in HCC and CCA tissues, which provide evidence that this miRNA undergoes down-regulation in liver cancer tissues. Moreover, the ability to detect miR-126 in the blood vessels of liver tissue sections concurs with previous studies that showed the specificity of miR-126 as an endothelial-miRNA (115,116). Interestingly, the results demonstrated that miR-126 also undergoes expression in the sinusoid cells of HCC tissues, which comprise capillary-like small blood vessels that fulfill a function in neoangiogenesis and tumor progression. One of the main characteristics of a liver tumor involves the presence of high vascular integrity as a sinusoid like tumor vessel formation, which is also known as sinusoidal capillarization. In contrast, normal liver sinusoid cells are differ morphologically and functionally from tumor sinusoid, for example, they do not usually express tumor markers such as CD34 and Willebrand Factor (vWF) (158,159). In 2001, Ohmori *et al.* reported that in comparison to the control group, CD34 was expressed at a higher level in the sinusoid-like tumor vessels of HCV-related HCC (159). This finding suggests that CD34 represents a strong predictor for HCC. Moreover, a study conducted by Du *et al.* showed that the overexpression of miR-126 in vivo reduced the expression of CD34

(134). This result indicates that miR-126 correlates inversely with CD34 expression, therefore reducing neoangiogenesis and tumor proliferation (134). The ISH result reveled an extensive expression of miR-126 in sinusoidal cells of the liver; however, our data on sinusoidal cells-miR-126 expression was not confirmed as non-tumor related sinusoid. Though, one can assume that normal sinusoids are miR-126 positive while transformed sinusoids exhibit a negative miR-126 expression. Further studies investigating miR-126 in sinusoid cells are needed to confirm this aspect.

Our results showed that miR-126 tend to be downregulated in cancer cells and tissues, which provide important evidence of miR-126 lose in liver cancer. The loss of miR-126 in hepatic carcinoma and cholangiocarcinoma cells and tissues provide an important perception of the involvement of miR-126 in malignant phenotype of liver cancer cells. Moreover, the deregulation of miR-126 in liver cancer cells and tissues indicate its role as a tumor suppressor gene.

4.1.2 Biological functions of miR-126 in HCC and CCA:

Studies have suggested that the down-regulation of miRNA in cancer acquired tumor suppressive properties. In the present study, I showed that miR-126 demonstrates marked down-regulation in HCC and CCA tissues relative to normal liver tissues and/or has a lower expression in HepG2 and HuccT1 cell lines relative toHEK293T. To test the hypothesis of miR-126 as a tumor suppressor, several gain of function analyses have been performed for analyzing the suppressive affect of miR-126 in tumor cells.

Numerous studies have revealed that miR-126 inhibits tumor cell growth, proliferation, and migration in different types of cancer, such as gastric and colon cancer (124-126,128). We proposed that increased expression of miR-126 in cancer cells effect liver cancer cells proliferation, migration and growth. Specifically, the present study showed a significant

correlation between miR-126 overexpression and tumor cell behavior. A cell viability assay (MTS) demonstrated that the restoration of miR-126 in HepG2 and HuccT1 cells has significantly reduced cell proliferation compared to the negative control group. Furthermore, in order to evaluate the effect of miR-126 on cell migration and growth, a scratch and colony formation assay was performed. As a result, these methods showed that the reintroduction of miR-126 in HepG2 and HuccT1 cells inhibited the ability of tumor cells to migrate and grow in colonies relative to the negative control group. Thus, the effect of miR-126 on cell growth and migration was statistically significant. Our data demonstrate a significant role of miR-126 in regulating several attributes of tumor cells behaviour. Indeed, from this result one can speculate that miR-126 impairs cancer progression not only by preventing cancer cell proliferation and growth but also by inhibiting cancer cells migration.

The mechanism by which miR-126 inhibits cell proliferation, migration, and growth remains unclear; however, several studies have attempted to illuminate these mechanisms. One of the early studies investigating miR-126 expression identified its host gene: the epidermal growth factor like domain 7 (EGFL7) (115,116,158) .A subsequent study suggested that the transcription of miR-126 and its host gene occur in parallel. This study also found that the mature sequence of miR-126 binds to the complementary sequence at the 3' end of EGFL7 and inhibits its mRNA translation, causing an overall reduction in EGFL7 levels (115,116). However, one of the main functions of EGFL7 induces the formation of new blood vessels from the pre-existing vessels, a process known as angiogenesis. Thus, miR-126 acts as a negative regulator on EGFL7 mRNA translation, which results in a reduction of the cell migration and angiogenesis processes (158). Recently, Liu *et al.* showed that the hyper-methylation of EGFL7 leads to the down-regulation of miR-126 in esophageal squamous cell carcinoma (ESCC) (159). These authors also demonstrated that the knockdown of DNA methyltransferases (DNMTs), the DNA methylation

enzyme, decreases EGFL7 methylation and subsequently increases miR-126 expression in ESCC cell lines (159). Furthermore, Liu *et al.* revealed that the knockdown of DNMTs reduces the expression of ADAM metallopeptidase domain 9 (ADAM9), a critical protein for cell-cell or cell-matrix interactions (159)in vitro, suggesting ADAM9 as another target for miR-126. Research proposed that ADAM9 activates the Epidermal Growth Factor Receptor (EGFR), which subsequently stimulates many cellular processes, such as extracellular signal regulated kinase (ERK) and ribosomal S6 kinase (RSK) pathways as well as the phosphatidylinositide 3-kinases (PI3K) - AKT signaling pathway (160). The activation of these pathways aids in controlling cell proliferation, apoptosis, migration, and metabolism in normal and neoplastic transformation (161).

To the best of the knowledge, only one study has been performed on HCC to identify miR-126 potential targets (134). This lone study reported PIK3R2, a PI3K regulatory subunit, as a direct target for miR-126(134). This finding displays consistency with a study conducted by Gue *et al*., who reported that in colon cancer, miR-126 mediates growth suppression by inhibiting the (128)PI3K signaling pathway(128). In addition, research has also proposed that the activation of Vascular Endothelial growth Factor (VEGF) negatively correlate with the effect of miR-126 on tumor cell proliferation and growth (162). VEGF is a cell-signaling protein that enhances the formation of new blood vessels. Liu *et al.* reported that the down-regulation of miR-126 in lung cancer cells induces the expression of VEGF and maintains tumor cell growth (162).

These studies illustrate that, in combination, the down-regulation of miR-126 may directly and indirectly activate EGFR, leading to the maintenance of growth and proliferation in tumor cells. Our data provides an intuition of the regulation of cancer cells proliferation, migration and growth by miR-126. Since miR-126 has been recognized to targets several growth factor such as VEGF, it is possible that miR-126 involved in cell homeostasis conservation by

control cell sensitivity to the growth factors important for tumor initiation and growth.

4.1.3 Summary and conclusion:

According to the most recent statistical estimations of cancer incidence, 3,2545,000 new cases have been reported in the past 5 years (GLOBOCAN Site, 2012). Liver cancer alone comprises 782,000 new cases, constituting 9.1% of total worldwide cancer mortality. The mortality of liver cancer mainly results from poor prognosis and the lack of effective treatment. However, the discovery of miRNA has provided a new promising direction in cancer research, as many miRNA have been established to control cell functions in health and disease; these molecules are now considered as cancer-associated genes. Much research has studied liver cancer, and many miRNA have been reported to modulate liver cancer progression and act as tumor suppressor genes or/and oncogenes. However, the tumor suppressor MiR-126 has not undergone significant research in HCC and CCA. Accordingly, the objective of this thesis aimed to study the expression and biological effect of miR-126 on HCC and CCA cell lines.

This study represented the first investigation showing that miR-126 is downregulated in the tumor cells of HCC and CCA tissue sections and demonstrates high expression in hepatocytes and blood vessels *in situ*. Furthermore, the thesis showed that the re-introduction of miR-126 in HepG2 and HuccT1 cell lines decreases cell proliferation, growth, and migration in comparison to the negative control group.

The data presented in this thesis encourages speculation of many assumptions and hypotheses. For instance, one can assume that the overexpression of miR-126 affects the behavior of tumor cells by targeting several growth factors, cellular receptors, and key pathway components, such as EGFR, VEGF, ADAM9, and PIK3R2. All of these molecules comprise critical factors that are mainly responsible for activating many signaling pathways that regulate cell proliferation, apoptosis, differentiation, and malignant transformation.

In conclusion, the tumor suppressive properties of miR-126 may offer a new insight in liver cancer therapy. Through the restoration of miR-126 levels, one may possess the ability to inhibit independent pathways that are predominantly responsible for tumor initiation, growth, and progression. Considering the importance of miR-126 in cancer pathogenesis, miR-126 may serve as a novel anti-cancer therapy.

4.1.4 Limitations and future directions:

The main limitation of this study involves the small number of samples. In the present study, only two cell lines were used: the HCC cell line HepG2 and the CCA cell line HuCCT1. Furthermore, only 3 cases of CCA and 2 cases of HCC were utilized. Consequently, future studies should implement larger sample sizes as well as include additional cases of CCA and HCC.

miRNA execute the post-transcriptional regulation of gene expression by targeting the 3'UTR of specific mRNA. In this thesis, the preliminary results suggested that miR-126 fulfills a critical role in the pathogenesis of HCC and CCA. However, the identification of miR-126 target gene was not applied in our study. Subsequent studies should aim to identify the target genes of miRNA-126 to understand the mechanisms of miRNA-126 gene silencing. Du *et al.* have reported PIK3R2 as a main target for miR-126 in HCC; however, further investigations are needed to confirm this finding. Moreover, a paucity of information exists regarding the role of MiR-126 in CCA, as, to the best of our knowledge, only one study, conducted by McNally *et al.*, has shown the deregulation of miR-126 in CCA tissue. More studies are required to examine the role of miR-126 in HCC and CCA by first identifying the target genes.

On the other hand, miRNA were intensively studied in different types of cancers. These investigations suggested that miRNA could function as a set of potential prognostic and

diagnostic markers. The *in situ* analysis demonstrated the down-regulation of miR-126 in HCC and CCA tissue; however, this investigation lacked access to a history of patients. Hence, future investigations should examine the correlation between miR-126 and the prognosis of liver cancer patients, thereby enabling the possibility of using miR-126 as prognostic and diagnostic biomarker in HCC and CCA.

Reference:

(1) McNally PR. GI/liver Secrets Plus. Philadelphia, PA: Mosby/Elsevier; 2010.

(2) Raza A, Sood GK. Hepatocellular carcinoma review: current treatment, and evidence-based medicine. World J Gastroenterol 2014 Apr 21;20(15):4115-4127.

(3) Poon RT, Fan ST, Lo CM, Ng IO, Liu CL, Lam CM, et al. Improving survival results after resection of hepatocellular carcinoma: a prospective study of 377 patients over 10 years. Ann Surg 2001 Jul;234(1):63-70.

(4) Venook AP, Papandreou C, Furuse J, de Guevara LL. The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective. Oncologist 2010;15 Suppl 4:5-13.

(5) Seeff LB, Hoofnagle JH. Epidemiology of hepatocellular carcinoma in areas of low hepatitis B and hepatitis C endemicity. Oncogene 2006 Jun 26;25(27):3771-3777.

(6) Fenoglio L, Serraino C, Castagna E, Cardellicchio A, Pomero F, Grosso M, et al. Epidemiology, clinical-treatment patterns and outcome in 256 hepatocellular carcinoma cases. World J Gastroenterol 2013 Jun 7;19(21):3207-3216.

(7) Fernandez-Rodriguez CM, Gutierrez-Garcia ML. Prevention of hepatocellular carcinoma in patients with chronic hepatitis B. World J Gastrointest Pharmacol Ther 2014 Aug 6;5(3):175-182.

(8) Kremsdorf D, Soussan P, Paterlini-Brechot P, Brechot C. Hepatitis B virus-related hepatocellular carcinoma: paradigms for viral-related human carcinogenesis. Oncogene 2006 Jun 26;25(27):3823-3833.

(9) Brechot C, Gozuacik D, Murakami Y, Paterlini-Brechot P. Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). Semin Cancer Biol 2000 Jun;10(3):211-231.

(10) Ayub A, Ashfaq UA, Haque A. HBV induced HCC: major risk factors from genetic to molecular level. Biomed Res Int 2013;2013:810461.

(11) Li X, Liu X, Tian L, Chen Y. Cytokine-Mediated Immunopathogenesis of Hepatitis B Virus Infections. Clin Rev Allergy Immunol 2014 Dec 6.

(12) Sinn DH, Gwak GY, Cho J, Paik SW, Yoo BC. Comparison of Clinical Manifestations and Outcomes between Hepatitis B Virus- and Hepatitis C Virus-Related Hepatocellular Carcinoma: Analysis of a Nationwide Cohort. PLoS One 2014 Nov 5;9(11):e112184.

(13) Koike K. Molecular basis of hepatitis C virus-associated hepatocarcinogenesis: lessons from animal model studies. Clin Gastroenterol Hepatol 2005 Oct;3(10 Suppl 2):S132-5.

(14) Sidharthan S, Kottilil S. Mechanisms of alcohol-induced hepatocellular carcinoma. Hepatol Int 2014 Sep;8(2):452-457.

(15) Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 1991 Apr 4;350(6317):427-428.

(16) Bruix J, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. Gut 2014 May;63(5):844-855.

(17) Zhu K, Dai Z, Zhou J. Biomarkers for hepatocellular carcinoma: progression in early diagnosis, prognosis, and personalized therapy. Biomark Res 2013 Feb 5;1(1):10-7771-1-10.

(18) Behne T, Copur MS. Biomarkers for hepatocellular carcinoma. Int J Hepatol 2012;2012:859076.

(19) Feng X, Xu R, Du X, Dou K, Qin X, Xu J, et al. Combination Therapy With Sorafenib and Radiofrequency Ablation for BCLC Stage 0-B1 Hepatocellular Carcinoma: A Multicenter Retrospective Cohort Study. Am J Gastroenterol 2014 Dec;109(12):1891-1899.

(20) Ebara M, Okabe S, Kita K, Sugiura N, Fukuda H, Yoshikawa M, et al. Percutaneous ethanol injection for small hepatocellular carcinoma: therapeutic efficacy based on 20-year observation. J Hepatol 2005 Sep;43(3):458-464.

(21) Bruix J, Sherman M, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. Hepatology 2011 Mar;53(3):1020-1022.

(22) Benson AB,3rd, D'Angelica MI, Abrams TA, Are C, Bloomston PM, Chang DT, et al. Hepatobiliary cancers, version 2.2014. J Natl Compr Canc Netw 2014 Aug;12(8):1152-1182.

(23) Mosconi S, Beretta GD, Labianca R, Zampino MG, Gatta G, Heinemann V. Cholangiocarcinoma. Crit Rev Oncol Hematol 2009 Mar;69(3):259-270.

(24) Gatto M, Bragazzi MC, Semeraro R, Napoli C, Gentile R, Torrice A, et al. Cholangiocarcinoma: update and future perspectives. Dig Liver Dis 2010 Apr;42(4):253-260.

(25) Cardinale V, Bragazzi MC, Carpino G, Torrice A, Fraveto A, Gentile R, et al. Cholangiocarcinoma: increasing burden of classifications. Hepatobiliary Surg Nutr 2013 Oct;2(5):272-280.

(26) Blechacz BR, Gores GJ. Cholangiocarcinoma. Clin Liver Dis 2008 Feb;12(1):131-50, ix.

(27) Kurathong S, Lerdverasirikul P, Wongpaitoon V, Pramoolsinsap C, Kanjanapitak A, Varavithya W, et al. Opisthorchis viverrini infection and cholangiocarcinoma. A prospective, case-controlled study. Gastroenterology 1985 Jul;89(1):151-156.

(28) Tesana S, Takahashi Y, Sithithaworn P, Ando K, Sakakura T, Yutanawiboonchai W, et al. Ultrastructural and immunohistochemical analysis of cholangiocarcinoma in immunized Syrian golden hamsters infected with Opisthorchis viverrini and administered with dimethylnitrosamine. Parasitol Int 2000 Sep;49(3):239-251.

(29) Sakpal SV, Babel N, Chamberlain RS. Surgical management of hepatolithiasis. HPB (Oxford) 2009 May;11(3):194-202.

(30) Fava G, Lorenzini I. Molecular pathogenesis of cholangiocarcinoma. Int J Hepatol 2012;2012:630543.

(31) Lazaridis KN, Gores GJ. Primary sclerosing cholangitis and cholangiocarcinoma. Semin Liver Dis 2006 Feb;26(1):42-51.

(32) Komori J, Marusawa H, Machimoto T, Endo Y, Kinoshita K, Kou T, et al. Activation-induced cytidine deaminase links bile duct inflammation to human cholangiocarcinoma. Hepatology 2008 Mar;47(3):888-896.

(33) Fevery J, Verslype C, Lai G, Aerts R, Van Steenbergen W. Incidence, diagnosis, and therapy of cholangiocarcinoma in patients with primary sclerosing cholangitis. Dig Dis Sci 2007 Nov;52(11):3123-3135.

(34) Rizzi PM, Ryder SD, Portmann B, Ramage JK, Naoumov NV, Williams R. p53 Protein overexpression in cholangiocarcinoma arising in primary sclerosing cholangitis. Gut 1996 Feb;38(2):265-268.

(35) Ahrendt SA, Rashid A, Chow JT, Eisenberger CF, Pitt HA, Sidransky D. p53 overexpression and K-ras gene mutations in primary sclerosing cholangitis-associated biliary tract cancer. J Hepatobiliary Pancreat Surg 2000;7(4):426-431.

(36) Ito T, Sakurai-Yageta M, Goto A, Pairojkul C, Yongvanit P, Murakami Y. Genomic and transcriptional alterations of cholangiocarcinoma. J Hepatobiliary Pancreat Sci 2014 Jun;21(6):380-387.

(37) Lozano E, Sanchez-Vicente L, Monte MJ, Herraez E, Briz O, Banales JM, et al. Cocarcinogenic effects of intrahepatic bile acid accumulation in cholangiocarcinoma development. Mol Cancer Res 2014 Jan;12(1):91-100.

(38) Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology 2013 Dec;145(6):1215-1229.

(39) Vilgrain V. Staging cholangiocarcinoma by imaging studies. HPB (Oxford) 2008;10(2):106-109.

(40) Lumachi F, Lo Re G, Tozzoli R, D'Aurizio F, Facomer F, Chiara GB, et al. Measurement of serum carcinoembryonic antigen, carbohydrate antigen 19-9, cytokeratin-19 fragment and matrix metalloproteinase-7 for detecting cholangiocarcinoma: a preliminary case-control study. Anticancer Res 2014 Nov;34(11):6663-6667.

(41) Vogel A, Wege H, Caca K, Nashan B, Neumann U. The diagnosis and treatment of cholangiocarcinoma. Dtsch Arztebl Int 2014 Oct 31;111(44):748-754.

(42) Patel AH, Harnois DM, Klee GG, LaRusso NF, Gores GJ. The utility of CA 19-9 in the diagnoses of cholangiocarcinoma in patients without primary sclerosing cholangitis. Am J Gastroenterol 2000 Jan;95(1):204-207.

(43) Liu RQ, Shen SJ, Hu XF, Liu J, Chen LJ, Li XY. Prognosis of the intrahepatic cholangiocarcinoma after resection: hepatitis B virus infection and adjuvant chemotherapy are favorable prognosis factors. Cancer Cell Int 2013 Oct 18;13(1):99-2867-13-99.

(44) Kim SW, Lim DH, Park HC, Park W, Park JO, Park YS. Salvage Radiation Therapy for Isolated Local Recurrence of Extrahepatic Cholangiocarcinoma After Radical Surgery: A Retrospective Study. Ann Surg Oncol 2014 Oct 16.

(45) DeOliveira ML. Liver transplantation for cholangiocarcinoma: current best practice. Curr Opin Organ Transplant 2014 Jun;19(3):245-252.

(46) Rosen CB, Heimbach JK, Gores GJ. Surgery for cholangiocarcinoma: the role of liver transplantation. HPB (Oxford) 2008;10(3):186-189.

(47) Kondo S, Takada T, Miyazaki M, Miyakawa S, Tsukada K, Nagino M, et al. Guidelines for the management of biliary tract and ampullary carcinomas: surgical treatment. J Hepatobiliary Pancreat Surg 2008;15(1):41-54.

(48) Rea DJ, Heimbach JK, Rosen CB, Haddock MG, Alberts SR, Kremers WK, et al. Liver transplantation with neoadjuvant chemoradiation is more effective than resection for hilar cholangiocarcinoma. Ann Surg 2005 Sep;242(3):451-8; discussion 458-61.

(49) Davis-Dusenbery BN, Hata A. Mechanisms of control of microRNA biogenesis. J Biochem 2010 Oct;148(4):381-392.

(50) Lee RC, Feinbaum RL, Ambrosts V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843-854.

(51) Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. Mutat Res 2011 Dec 1;717(1-2):1-8.

(52) Macfarlane LA, Murphy PR. MicroRNA: Biogenesis, Function and Role in Cancer. Curr Genomics 2010 Nov;11(7):537-561.

(53) Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell 2003 Dec 26;115(7):787-798.

(54) Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009 Feb;10(2):126-139.

(55) Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J 2004 Oct 13;23(20):4051-4060.

(56) Gromak N. Intronic microRNAs: a crossroad in gene regulation. Biochem Soc Trans 2012 Aug;40(4):759-761.

(57) Zhuo Y, Gao G, Shi JA, Zhou X, Wang X. miRNAs: biogenesis, origin and evolution, functions on virus-host interaction. Cell Physiol Biochem 2013;32(3):499-510.

(58) Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome Res 2004 Oct;14(10A):1902-1910.

(59) Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 2002 Sep 2;21(17):4663-4670.

(60) Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science 2004 Jan 2;303(5654):95-98.

(61) Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 2004 Dec 15;18(24):3016-3027.

(62) Landthaler M, Yalcin A, Tuschl T. The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. Curr Biol 2004 Dec 14;14(23):2162-2167.

(63) Muralidhar B, Goldstein LD, Ng G, Winder DM, Palmer RD, Gooding EL, et al. Global microRNA profiles in cervical squamous cell carcinoma depend on Drosha expression levels. J Pathol 2007 Aug;212(4):368-377.

(64) Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, et al. The Microprocessor complex mediates the genesis of microRNAs. Nature 2004 Nov 11;432(7014):235-240.

(65) Bohnsack MT, Czaplinski K, Gorlich D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA 2004 Feb;10(2):185-191.

(66) Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 2003 Dec 15;17(24):3011-3016.

(67) Melo SA, Ropero S, Moutinho C, Aaltonen LA, Yamamoto H, Calin GA, et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. Nat Genet 2009 Mar;41(3):365-370.

(68) Haase AD, Jaskiewicz L, Zhang H, Laine S, Sack R, Gatignol A, et al. TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep 2005 Oct;6(10):961-967.

(69) Heo I, Joo C, Cho J, Ha M, Han J, Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. Mol Cell 2008 Oct 24;32(2):276-284.

(70) Mayr F, Heinemann U. Mechanisms of Lin28-mediated miRNA and mRNA regulation--a structural and functional perspective. Int J Mol Sci 2013 Aug 9;14(8):16532-16553.

(71) Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 2009 Mar;11(3):228-234.

(72) Hock J, Meister G. The Argonaute protein family. Genome Biol 2008;9(2):210-2008-9-2-210. Epub 2008 Feb 26.

(73) Kuhn CD, Joshua-Tor L. Eukaryotic Argonautes come into focus. Trends Biochem Sci 2013 May;38(5):263-271.

(74) Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell 2004 Jul 23;15(2):185-197.

(75) Song JJ, Liu J, Tolia NH, Schneiderman J, Smith SK, Martienssen RA, et al. The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nat Struct Biol 2003 Dec;10(12):1026-1032.

(76) Lingel A, Simon B, Izaurralde E, Sattler M. Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. Nat Struct Mol Biol 2004 Jun;11(6):576-577.

(77) Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev 2006 Jul 15;20(14):1885-1898.

(78) Behm-Ansmant I, Rehwinkel J, Izaurralde E. MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. Cold Spring Harb Symp Quant Biol 2006;71:523-530.

(79) Chendrimada TP, Finn KJ, Ji X, Baillat D, Gregory RI, Liebhaber SA, et al. MicroRNA silencing through RISC recruitment of eIF6. Nature 2007 Jun 14;447(7146):823-828.

(80) Humphreys DT, Westman BJ, Martin DI, Preiss T. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci U S A 2005 Nov 22;102(47):16961-16966.

(81) Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A 2004 Mar 2;101(9):2999-3004.

(82) Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2002 Nov 26;99(24):15524-15529.

(83) He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, et al. A microRNA polycistron as a potential human oncogene. Nature 2005 Jun 9;435(7043):828-833.

(84) Olive V, Li Q, He L. Mir-17-92: a Polycistronic Oncomir with Pleiotropic Functions. Immunol Rev 2013 May;253(1):158-166.

(85) Ota A, Tagawa H, Karnan S, Tsuzuki S, Karpas A, Kira S, et al. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res 2004 May 1;64(9):3087-3095.

(86) van Haaften G, Agami R. Tumorigenicity of the miR-17-92 cluster distilled. Genes Dev 2010 Jan 1;24(1):1-4.

(87) Diosdado B, van de Wiel MA, Terhaar Sive Droste JS, Mongera S, Postma C, Meijerink WJ, et al. MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression. Br J Cancer 2009 Aug 18;101(4):707-714.

(88) Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, et al. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res 2005 Nov 1;65(21):9628-9632.

(89) Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005 Aug 15;65(16):7065-7070.

(90) Michael MZ, O'Connor SM, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 2003 Oct;1(12):882-891.

(91) Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature 2005 Jun 9;435(7043):834-838.

(92) Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 2006 Feb 14;103(7):2257-2261.

(93) Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 2005 Sep 27;102(39):13944-13949.

(94) Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 2004 Jun 1;64(11):3753-3756.

(95) Donnem T, Eklo K, Berg T, Sorbye SW, Lonvik K, Al-Saad S, et al. Prognostic impact of MiR-155 in non-small cell lung cancer evaluated by in situ hybridization. J Transl Med 2011 Jan 10;9:6-5876-9-6.

(96) Gironella M, Seux M, Xie MJ, Cano C, Tomasini R, Gommeaux J, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. Proc Natl Acad Sci U S A 2007 Oct 9;104(41):16170-16175.

(97) Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci U S A 2005 Mar 8;102(10):3627-3632.

(98) Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol 2002 Apr 30;12(9):735-739.

(99) Xiao F, Zhang W, Zhou L, Xie H, Xing C, Ding S, et al. microRNA-200a is an independent prognostic factor of hepatocellular carcinoma and induces cell cycle arrest by targeting CDK6. Oncol Rep 2013 Nov;30(5):2203-2210.

(100) Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J. miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis 2010 May;31(5):766-776.

(101) Lin CJ, Gong HY, Tseng HC, Wang WL, Wu JL. miR-122 targets an anti-apoptotic gene, Bcl-w, in human hepatocellular carcinoma cell lines. Biochem Biophys Res Commun 2008 Oct 24;375(3):315-320.

(102) Yang X, Liang L, Zhang XF, Jia HL, Qin Y, Zhu XC, et al. MicroRNA-26a suppresses tumor growth and metastasis of human hepatocellular carcinoma by targeting interleukin-6-Stat3 pathway. Hepatology 2013 Jul;58(1):158-170.

(103) Gong J, Zhang JP, Li B, Zeng C, You K, Chen MX, et al. MicroRNA-125b promotes apoptosis by regulating the expression of Mcl-1, Bcl-w and IL-6R. Oncogene 2013 Jun 20;32(25):3071-3079.

(104) Jiang J, Gusev Y, Aderca I, Mettler TA, Nagorney DM, Brackett DJ, et al. Association of MicroRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. Clin Cancer Res 2008 Jan 15;14(2):419-427.

(105) Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 2006 Apr 20;25(17):2537-2545.

(106) Kawahigashi Y, Mishima T, Mizuguchi Y, Arima Y, Yokomuro S, Kanda T, et al. MicroRNA profiling of human intrahepatic cholangiocarcinoma cell lines reveals biliary epithelial cell-specific microRNAs. J Nippon Med Sch 2009 Aug;76(4):188-197.

(107) Lu L, Byrnes K, Han C, Wang Y, Wu T. miR-21 targets 15-PGDH and promotes cholangiocarcinoma growth. Mol Cancer Res 2014 Jun;12(6):890-900.

(108) Chusorn P, Namwat N, Loilome W, Techasen A, Pairojkul C, Khuntikeo N, et al. Overexpression of microRNA-21 regulating PDCD4 during tumorigenesis of liver fluke-associated cholangiocarcinoma contributes to tumor growth and metastasis. Tumour Biol 2013 Jun;34(3):1579-1588.

(109) Selaru FM, Olaru AV, Kan T, David S, Cheng Y, Mori Y, et al. MicroRNA-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3. Hepatology 2009 May;49(5):1595-1601.

(110) Peng F, Jiang J, Yu Y, Tian R, Guo X, Li X, et al. Direct targeting of SUZ12/ROCK2 by miR-200b/c inhibits cholangiocarcinoma tumourigenesis and metastasis. Br J Cancer 2013 Dec 10;109(12):3092-3104.

(111) Hu C, Huang F, Deng G, Nie W, Huang W, Zeng X. miR-31 promotes oncogenesis in intrahepatic cholangiocarcinoma cells via the direct suppression of RASA1. Exp Ther Med 2013 Nov;6(5):1265-1270.

(112) Kuehbacher A, Urbich C, Zeiher AM, Dimmeler S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. Circ Res 2007 Jul 6;101(1):59-68.

(113) Suarez Y, Fernandez-Hernando C, Pober JS, Sessa WC. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. Circ Res 2007 Apr 27;100(8):1164-1173.

(114) Yang WJ, Yang DD, Na S, Sandusky GE, Zhang Q, Zhao G. Dicer is required for embryonic angiogenesis during mouse development. J Biol Chem 2005 Mar 11;280(10):9330-9335.

(115) Meister J, Schmidt MH. miR-126 and miR-126*: new players in cancer. ScientificWorldJournal 2010 Oct 12;10:2090-2100.

(116) Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, et al. miR-126 regulates angiogenic signaling and vascular integrity. Dev Cell 2008 Aug;15(2):272-284.

(117) Wei Y, Nazari-Jahantigh M, Neth P, Weber C, Schober A. MicroRNA-126, -145, and -155: a therapeutic triad in atherosclerosis? Arterioscler Thromb Vasc Biol 2013 Mar;33(3):449-454.

(118) Nazari-Jahantigh M, Egea V, Schober A, Weber C. MicroRNA-specific regulatory mechanisms in atherosclerosis. J Mol Cell Cardiol 2014 Nov 8.

(119) Hong F, Li Y, Xu Y. Decreased placental miR-126 expression and vascular endothelial growth factor levels in patients with pre-eclampsia. J Int Med Res 2014 Dec;42(6):1243-1251.

(120) Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, et al. Endogenous human microRNAs that suppress breast cancer metastasis. Nature 2008 Jan 10;451(7175):147-152.

(121) Vosa U, Vooder T, Kolde R, Vilo J, Metspalu A, Annilo T. Meta-analysis of microRNA expression in lung cancer. Int J Cancer 2013 Jun 15;132(12):2884-2893.

(122) Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006 Mar;9(3):189-198.

(123) Liu B, Peng XC, Zheng XL, Wang J, Qin YW. MiR-126 restoration down-regulate VEGF and inhibit the growth of lung cancer cell lines in vitro and in vivo. Lung Cancer 2009 Nov;66(2):169-175.

(124) Feng R, Chen X, Yu Y, Su L, Yu B, Li J, et al. miR-126 functions as a tumour suppressor in human gastric cancer. Cancer Lett 2010 Dec 1;298(1):50-63.

(125) Otsubo T, Akiyama Y, Hashimoto Y, Shimada S, Goto K, Yuasa Y. MicroRNA-126 inhibits SOX2 expression and contributes to gastric carcinogenesis. PLoS One 2011 Jan 27;6(1):e16617.

(126) Li Z, Li N, Wu M, Li X, Luo Z, Wang X. Expression of miR-126 suppresses migration and invasion of colon cancer cells by targeting CXCR4. Mol Cell Biochem 2013 Sep;381(1-2):233-242.

(127) Li XM, Wang AM, Zhang J, Yi H. Down-regulation of miR-126 expression in colorectal cancer and its clinical significance. Med Oncol 2011 Dec;28(4):1054-1057.

(128) Guo C, Sah JF, Beard L, Willson JK, Markowitz SD, Guda K. The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers. Genes Chromosomes Cancer 2008 Nov;47(11):939-946.

(129) Zhang Y, Li M, Wang H, Fisher WE, Lin PH, Yao Q, et al. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. World J Surg 2009 Apr;33(4):698-709.

(130) McNally ME, Collins A, Wojcik SE, Liu J, Henry JC, Jiang J, et al. Concomitant dysregulation of microRNAs miR-151-3p and miR-126 correlates with improved survival in resected cholangiocarcinoma. HPB (Oxford) 2013 Apr;15(4):260-264.

(131) Novellino L, Rossi RL, Bonino F, Cavallone D, Abrignani S, Pagani M, et al. Circulating hepatitis B surface antigen particles carry hepatocellular microRNAs. PLoS One 2012;7(3):e31952.

(132) Chen H, Miao R, Fan J, Han Z, Wu J, Qiu G, et al. Decreased expression of miR-126 correlates with metastatic recurrence of hepatocellular carcinoma. Clin Exp Metastasis 2013 Jun;30(5):651-658.

(133) Han ZB, Zhong L, Teng MJ, Fan JW, Tang HM, Wu JY, et al. Identification of recurrence-related microRNAs in hepatocellular carcinoma following liver transplantation. Mol Oncol 2012 Aug;6(4):445-457.

(134) Du C, Lv Z, Cao L, Ding C, Gyabaah OA, Xie H, et al. MiR-126-3p suppresses tumor metastasis and angiogenesis of hepatocellular carcinoma by targeting LRP6 and PIK3R2. J Transl Med 2014 Sep 22;12:259-014-0259-1.

(135) Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology 2008 Jun;47(6):1955-1963.

(136) Jiang F, Zhao W, Zhou L, Liu Z, Li W, Yu D. MiR-222 Targeted PUMA to Improve Sensitization of UM1 Cells to Cisplatin. Int J Mol Sci 2014 Dec 2;15(12):22128-22141.

(137) Wang Z, Ma X, Cai Q, Wang X, Yu B, Cai Q, et al. MiR-199a-3p promotes gastric cancer progression by targeting ZHX1. FEBS Lett 2014 Oct 27;588(23):4504-4512.

(138) Pei H, Jin Z, Chen S, Sun X, Yu J, Guo W. MiR-135b promotes proliferation and invasion of osteosarcoma cells via targeting FOXO1. Mol Cell Biochem 2014 Nov 22.

(139) Li P, Xu Q, Zhang D, Li X, Han L, Lei J, et al. Upregulated miR-106a plays an oncogenic role in pancreatic cancer. FEBS Lett 2014 Mar 3;588(5):705-712.

(140) Zhu M, Zhang N, He S, Lui Y, Lu G, Zhao L. MicroRNA-106a targets TIMP2 to regulate invasion and metastasis of gastric cancer. FEBS Lett 2014 Feb 14;588(4):600-607.

(141) Humphreys KJ, McKinnon RA, Michael MZ. miR-18a Inhibits CDC42 and Plays a Tumour Suppressor Role in Colorectal Cancer Cells. PLoS One 2014 Nov 7;9(11):e112288.

(142) Hershkovitz-Rokah O, Modai S, Pasmanik-Chor M, Toren A, Shomron N, Raanani P, et al. MiR-30e induces apoptosis and sensitizes K562 cells to imatinib treatment via regulation of the BCR-ABL protein. Cancer Lett 2015 Jan 28;356(2 Pt B):597-605.

(143) Zhao X, Duan Z, Liu X, Wang B, Wang X, He J, et al. MicroRNA-127 is downregulated by Tudor-SN protein and contributes to metastasis and proliferation in breast cancer cell line MDA-MB-231. Anat Rec (Hoboken) 2013 Dec;296(12):1842-1849.

(144) Pekarsky Y, Santanam U, Cimmino A, Palamarchuk A, Efanov A, Maximov V, et al. Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. Cancer Res 2006 Dec 15;66(24):11590-11593.

(145) Mott JL, Kurita S, Cazanave SC, Bronk SF, Werneburg NW, Fernandez-Zapico ME. Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB. J Cell Biochem 2010 Aug 1;110(5):1155-1164.

(146) Yang Z, Zhang Y, Wang L. A feedback inhibition between miRNA-127 and TGFbeta/c-Jun cascade in HCC cell migration via MMP13. PLoS One 2013 Jun 7;8(6):e65256.

(147) Wang Q, Tang H, Yin S, Dong C. Downregulation of microRNA-138 enhances the proliferation, migration and invasion of cholangiocarcinoma cells through the upregulation of RhoC/p-ERK/MMP-2/MMP-9. Oncol Rep 2013 May;29(5):2046-2052.

(148) Lin L, Liang H, Wang Y, Yin X, Hu Y, Huang J, et al. microRNA-141 inhibits cell proliferation and invasion and promotes apoptosis by targeting hepatocyte nuclear factor-3beta in hepatocellular carcinoma cells. BMC Cancer 2014 Nov 25;14(1):879-2407-14-879.

(149) Korhan P, Erdal E, Atabey N. MiR-181a-5p is downregulated in hepatocellular carcinoma and suppresses motility, invasion and branching-morphogenesis by directly targeting c-Met. Biochem Biophys Res Commun 2014 Aug 8;450(4):1304-1312.

(150) Tan S, Li R, Ding K, Lobie PE, Zhu T. miR-198 inhibits migration and invasion of hepatocellular carcinoma cells by targeting the HGF/c-MET pathway. FEBS Lett 2011 Jul 21;585(14):2229-2234.

(151) Qiu YH, Wei YP, Shen NJ, Wang ZC, Kan T, Yu WL, et al. miR-204 inhibits epithelial to mesenchymal transition by targeting slug in intrahepatic cholangiocarcinoma cells. Cell Physiol Biochem 2013;32(5):1331-1341.

(152) Li B, Han Q, Zhu Y, Yu Y, Wang J, Jiang X. Down-regulation of miR-214 contributes to intrahepatic cholangiocarcinoma metastasis by targeting Twist. FEBS J 2012 Jul;279(13):2393-2398.

(153) Ni F, Zhao H, Cui H, Wu Z, Chen L, Hu Z, et al. MicroRNA-362-5p promotes tumor growth and metastasis by targeting CYLD in hepatocellular carcinoma. Cancer Lett 2015 Jan 28;356(2 Pt B):809-818.

(154) Xue J, Chen LZ, Li ZZ, Hu YY, Yan SP, Liu LY. MicroRNA-433 inhibits cell proliferation in hepatocellular carcinoma by targeting p21 activated kinase (PAK4). Mol Cell Biochem 2014 Nov 20.

(155) Huang XP, Hou J, Shen XY, Huang CY, Zhang XH, Xie YA, et al. Microrna-486-5p, downregulated in hepatocelluar carcinoma, suppresses tumor growth by targeting p85alpha. FEBS J 2014 Dec 5.

(156) Li J, Tian F, Li D, Chen J, Jiang P, Zheng S, et al. MiR-605 represses PSMD10/Gankyrin and inhibits intrahepatic cholangiocarcinoma cell progression. FEBS Lett 2014 Sep 17;588(18):3491-3500.

(157) Geback T, Schulz MM, Koumoutsakos P, Detmar M. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. BioTechniques 2009 Apr;46(4):265-274.

(158) Nikolic I, Plate KH, Schmidt MH. EGFL7 meets miRNA-126: an angiogenesis alliance. J Angiogenes Res 2010 Jun 8;2(1):9-2384-2-9.

(159) Liu R, Gu J, Jiang P, Zheng Y, Liu X, Jiang X, et al. DNMT1-microRNA126 epigenetic circuit contributes to esophageal squamous cell carcinoma growth via ADAM9-EGFR-AKT signaling. Clin Cancer Res 2014 Dec 15.

(160) Fischer OM, Hart S, Gschwind A, Ullrich A. EGFR signal transactivation in cancer cells. Biochem Soc Trans 2003 Dec;31(Pt 6):1203-1208.

(161) Er EE, Mendoza MC, Mackey AM, Rameh LE, Blenis J. AKT facilitates EGFR trafficking and degradation by phosphorylating and activating PIKfyve. Sci Signal 2013 Jun 11;6(279):ra45.

(162) Suarez Y, Fernandez-Hernando C, Yu J, Gerber SA, Harrison KD, Pober JS, et al. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. Proc Natl Acad Sci U S A 2008 Sep 16;105(37):14082-14087.