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IDENTIFICATION OF PUTATIVE MicroRNAs FOR EARLY DETECTION OF HEPATOCARCINOMA

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ABSTRACT

Hepatocellular carcinoma is most prevalent and life threatening cancer. Its early diagnosis and metastasis monitoring are of the utmost importance. With the advance in understanding of tumor biology along with advance development of cellular and molecular techniques, the role of biomarkers related to early detection, invasiveness, metastasis and recurrence has increased the interest of research resulting in discovery and utilization of several novel markers. In present review, cellular and molecular changes in HCC along with some reported serum marker including miRNAs are summarized. Furthermore tumor suppressive and tumor promoting roles and underlying mechanism of miRNAs in HCC are discussed.

KEYWORDS: Hepatocellular carcinoma, MicroRNA, Alpha fetoprotein, Phosphoinositide 3 kinase (PI3K).

INTRODUCTION

Hepatocellular carcinoma is one of the most common malignancies.[1] According to a study conducted in India by verbal autopsy in 1.1 million homes, the age standardized mortality rate for HCC in India for men is 6.8/100,000 and for women is 5.1/100,00[2] and incidence of HCC in cirrhotics in India is 1.6% per year.[3] HCC is a multistep process and its early diagnosis and metastasis monitoring are utmost required. The risk factor associated with the development of HCC are include hepatitis B and C virus infection[4] and other non viral factors include excessive alcohol consumption, exposure to environmental toxin like alpha

toxin B, hemochromatosis, cirrhosis, diabetes and obesity.[5] Many of these factors are known causes of chronic hepatitis (CH) and liver cirrhosis, which represent a pre-neoplastic condition of HCC.[6] HCC is often far advanced and may have multiple lesions at the time of diagnosis. Curative resection cannot be expected in case of extra hepatic metastases. In cases without extrahepatic metastases, curative resection could potentially be performed. However, postoperative recurrence and intrahepatic metastases occur frequently reducing the postoperative survival rate significantly.[6] It is believed that early detection of HCC before its metastasis will provide significant therapeutic tool to deal with it. Major pathways implicated in hepatocarcinogenesis include the RAF/MEK/ERK, phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), WNT/β-catenin, insulin like growth factor, hepatocyte growth factor/c-MET and growth factor regulated angiogenic signalling pathways.[7]

CELLULAR AND MOLECULAR ALTERATIONS IN HCC

Some major cellular signaling pathways have been implicated in HCC which are described below.[7, 8]

Wnt/β-catenin pathway

This pathway has fundamental role in embryogenesis as well as cell differentiation, proliferation and apoptosis. In the absence of Wnt signaling, cytoplasmic β catenin form complexes with APC, Axin1 and GSK-3β which phosphorylates β-catenin and target it for ubiquitiniation. In the event that Wnt signaling receptors are engaged, conformational change occur in Axin complex cause the release of β-catenin, which then localizes to the nucleus and activates the transcription of Myc, cyclin D1 and COX2 amongst others.[9] This signalling pathway is critically involved in liver development and postnatal liver homeostasis and also associated with many other important liver functions, such as ammonia and nitrogen metabolism, bile acid homeostasis, drug detoxification and injury recovery. Studies have shown that 50-70% of liver tumors have increased levels of β-catenin in the cytoplasm and in the nucleus which provide tumor cells a growth advantage.[10] Accumulation and stabilization of β-catenin could be a direct result from point mutations or deletions in the β-catenin gene, which is found in 12-26% of HCC.[11]

MAPK pathway

The intracellular mitogen activated protein kinase (MAPK) family has five MAPK subgroups. These include the extracellular signal-regulated kinase protein homologs 1 and 2

(ERK1/2), big MAPK-1 (BMK-1/ERK5), c-Jun N-terminal kinase homologs 1, 2, and 3 (JNK1/2/3), stress-activated protein kinase 2 (SAPK-2) homolog and (p38α/β/δ), and ERK6. The activity of these kinases is dependent upon dual phosphorylation of T and Y residues located in their activation loop. Proteins of HBV, HCV, and hepatitis E virus modulate MAPK signaling by targeting multiple steps along the signaling pathway. For instance, HCV E2 protein activates the MAPK pathway in human hepatoma Huh-7 cells and promotes cell proliferation. In human HCC, the expression levels of Spred protein (Sprouty-related protein with Ena/vasodilator-stimulated phosphoprotein homology 1 domain), an inhibitor of the Ras/Raf-1/ERK pathway are deregulated. Forced expression of Spred inhibits ERK activation in *vivo* and in *vitro*, resulting in reduced proliferation of cancer cells and low secretion of matrix metalloproteinase 2 and 9. This finding shows direct correlation of MAPK-ERK pathway activation and HCC, suggesting that Spred could serve as a therapeutic target for human HCC. Mutation in the RAS gene has been reported in 10-30% of HCC tumors. There is strong correlation between activated JNK signaling and HCC. For example, one study has shown that JNK1 is over-activated in 17 out of 31 samples (55%) from Chinese HCC patients. JNK1 knockout mice had a significant reduction in liver tumorigenesis chemically induced by DEN and hepatocyte proliferation also decreased in those animals. It was proposed that mice lacking JNK1 have increased expression of p21, a cell cycle inhibitor. In summary, this clearly demonstrated the deep involvement of MAPK signaling pathways during liver carcinogenesis.[11, 12]

PI3k/AKT/mTOR pathway

Phosphoinositide 3 kinase (PI3K) is an intracellular signal transducer enzyme that can phosphorylate the hydroxyl group of phosphatidylinositol. It belongs to a large family of PI3K-related kinase(PI3K) which produce the lipid second messenger phosphatidylinositol phosphate, which is absent in resting cells but can be acutely produced in response to activated PI3K. Akt is a serine-threonine kinase downstream of PI3K. Upon PI3K activation and phosphatidylinosoltriphosphate production, Akt is recruited to the plasma membrane through its PH domain together with another PH domain containing protein phosphoinositide dependent kinase (PDK1) which phosphorylates key residues in the kinase domain activation loop of Akt to activate Akt kinase activity. Activated Akt phosphorylates multiple protein substrates and regulates a variety of critical cellular activities. mTOR is a serine-threonine protein kinase that also belongs to PI3K-related kinase family which controls several important cellular processes including regulation of protein translation. mTOR enhances

translation initiation via two major targets, the eIF4E binding proteins (4E-BPs) and the ribosomal protein S6 kinases (S6K1 and S6K2). For HCC, one study has shown over expression of phospho-mTOR in 15% of liver tumors. Elevated Akt phosphorylation was also found in 23% of HCC and implicated early HCC recurrence and poor prognosis. There is also a high frequency (35.6%) of somatic PI3K mutations in HCC specimens. PTEN, Phhosphtase and tensin homolg which dephosphorylate the Phosphotidylinsoltriphosphate, the negative component of the PI3K/Akt/mTOR, is mutated in 5% of HCC and its expression is reduced in half of all HCC tumors, leading to the over activation of the pathway.[8, 11] In HCC patients, reduced PTEN expression has been associated with advanced tumor stage, high recurrences rate and poor survival outcome, suggesting inactivation of PTEN is involved in the pathogenesis of HCC.

p53 Pathway

As a major "guardian of the genome," the *p53* tumor suppressor gene plays a pivotal role in cell cycle control, apoptosis, and maintenance of genomic stability.[13] Several studies have reported that p53 mutations and inactivation play a critical role in HCC. For instance, in a clinical study of 16 Chinese patients with HCC, 8 had a point mutation at the third base position of codon 249. Moreover, the G to T transversion in seven HCC DNA samples and the G3C transversion in the other HCC were consistent with mutations caused by AFB1 in mutagenesis experiments where in serum hepatitis B surface antigen and liver AFB1-DNA adducts were found to be significantly elevated in HCC samples compared with controls.[14] Epidemiologically p53 mutation was frequently found in aflatoxin-induced HCC (~50%), but was rare in HCC that was not induced by aflatoxin (28-42%). In a study of hepatitis B and C, the p53 mutation profile was different for both as in HBV-related HCC (45%) was significantly higher than that 13% in HCV-related HCC.[11]

pRb Pathway

The tumor suppressor retinoblastoma protein pRb1 is a major cellular barrier to cancer development. It controls cell cycle progression via repression of the E2F transcription factor family of proteins.[15] The activity of cyclin-dependent kinases (CDKs) correlates with the onset of pRb phosphorylation and G1/S cell cycle transition. Up to 16 CDK phosphorylation sites exist on pRb. Several studies have demonstrated that the pRb pathway is severely disrupted in HCC patients demonstrating that pRB is a critical player in carcinogenesis. The

p16 gene, also known as cyclin dependent kinase inhibitor, is the regulator of the Rb pathway. Inactivation of either Rb or p16 was frequently found in HCC (81%).[11]

Ras Pathway

Human ras proteins H-Ras, N-Ras, K-ras4 A, and K-Ras 4B are small GTP-binding proteins that function as molecular switches to influence cell growth, differentiation and apoptosis. Single point mutations in codon 13 of H-ras, codon 12 of N-ras, and codon 61 of K-ras were originally observed in HCC caused by various chemicals such as N-nitrosomorpholin, bleomycin, 1-nitropyrene and methyl (acetoxymethyl) nitrosamine. Ras interacts with a downstream serine/threonine kinase Raf-1 leading to its activation and downstream signaling which includes activation of MAPK kinases MEK1 and MEK2, to regulate proliferation and apoptosis. [16] It has been suggested that the Ras pathway is important in HCC of rodents but not human HCC based on the low mutation rate of Ras in humans. However, in a recent study, it was reported that RASSF1A and NORE1A, members of the RASSF family of Ras inhibitors, are inactivated in human HCC, demonstrating the role for Ras pathway in liver cancer.

Growth factors and their receptors

The main risk factor for HCC is cirrhosis and several lines of evidence implicate epidermal growth factor (EGF) in the progression of cirrhosis and development of HCC.[17] Epidermal growth factor receptor system plays a central hepato-protective and pro-regenerative role in liver. Transforming growth factor-α (TGF-α) is an important autocrine growth regulator of hepatocytes that plays a role in development of hepatocellular carcinoma (HCC) among patients with chronic hepatitis C.[18] TGF-α stimulates the proliferation of HCC cells by activating the epidermal growth factor receptor signaling pathway. Mutation of the IGF-2 receptor was frequently found (25-55%) in HCC.[11] Alteration of this receptor is related to the over expression of mitogen IGF-2, because the receptor induces the degradation of IGF-2. The IGF-2 receptor also activates transforming growth factor-β (TGF-β), a negative regulator of cell growth, by binding to the latent complex of TGF-β. Alteration of the TGF-β receptor type II gene itself was also found in HCC (~10%).

Telomerase activity

Telomere is a region of repetitive DNA at the end of each chromosome, which contributes to the stability and integrity of the chromosome. The length of the telomere is maintained by the activity of telomerase, which is a ribonucleoprotein complex composed of telomerase reverse

transcriptase (TERT) and an RNA primer sequence.[19] Without TERT, the length of the telomere gradually decreases. If the cells divide without telomeres, they would lose the end of their chromosomes that contain necessary information. Thus, the length of the telomere limits the lifespan of normal somatic cells, TERT activity has been found in most human cancers. Activation of telomerase was frequently (~90%) found in HCC.[11] The maintenance of telomere stability seems to be required for the immortalization of cancer cells (Fig. 1).

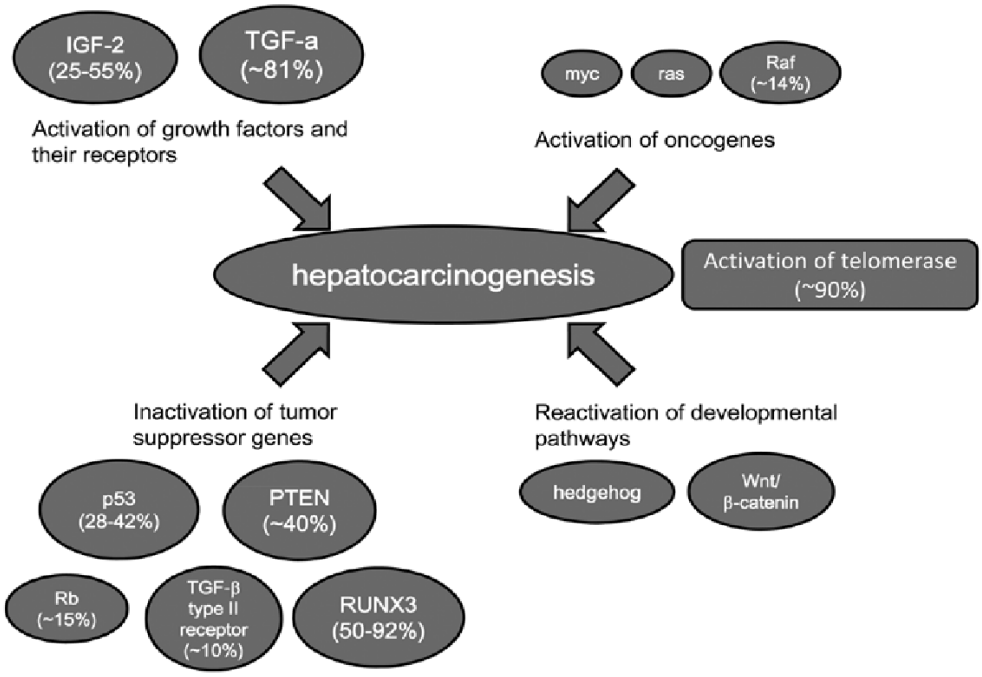

Figure 1: Gene alteration occurring in HCC[11]

BIOMARKERS

According to the US National Institute of Health's (NIH) Working Group and the Biomarkers Consortium, a biomarker is a characteristic that is objectively measured as an indicator of normal biological processes, pathogenic process or a pharmacological response to a therapeutic intervention (http://www.biomarkersconsortium.org). The United nation' World Health Organization (WHO) defines a biomarker as any substance, structure or process that can be measured in the body or its products and influences or predict the incidence of outcome or disease (Biomarkers in Risk Assessment: Validity and Validation, Environmental Health Criteria Series, No222, WHO).

Biomarkers of HCC

High morbidity observed in HCC majorly due to lack of early detection maker and poor prognosis which limits the option for chemotherapy, adjuvant therapy or surgical procedures.

With the advance in understanding of tumor biology along with advance development of cellular and molecular techniques, the role of biomarkers related to early detection, invasiveness, metastasis and recurrence has increased the interest of research resulting in discovery and utilization of several novel markers.[20] The ideal biomarker should fit a number of criteria depending on how the biomarker is to be used. It should be accessible through non-invasive methods, specific to the disease or pathology of interest, a reliable indication of disease before clinical symptoms appear (early detection), sensitive to changes in the pathology (disease progression or therapeutic response), and easily translatable from model systems to humans. Secreted miRNAs have many requisite features of good biomarkers. miRNAs are stable in various bodily fluids, the sequences of most miRNAs are conserved among different species, the expression of some miRNAs is specific to tissues or biological stages, and the level of miRNAs can be easily accessed by various methods, including methods such as polymerase chain reaction.[21]

Oncofetal and Glycoprotein Antigens

Alpha fetoprotein

Alpha fetoprotein is first serological assay for detection and clinical follow up for HCC. Serum AFP level are often elevated in HCC. But this is not always case, it may be drop or normalize as disease progress. In 1984, Chen *et al.* reported that serum AFP level more than 400 ng/mL is considered diagnostic; however, such high values are observed only in a small percentage of patients with HCC. Sensitivity and specificity of AFP vary widely, and total AFP is not always specific, especially early stages of HCC.[22] In general consistently elevated serum AFP levels greater than 500 ng/mL are indicative of HCC. Unfortunately, AFP serum concentrations do not correlate well with the prognostic values of HCC such as tumor size, stage, or disease progression.

Glypican-3(GPC-3)

GPC-3 , a membrane anchored heparin sulphate proteoglycan which normally expressed in fetal liver only.[23] It interacts with growth factors and modulates their activities. It binds to the cell membrane through the glycosyl phosphatidylinositol anchors. The expression of GPC3 (mRNA and protein level) was up regulated significantly in tumor tissues of HCC compared to paraneoplastic liver tissue, liver tissues of healthy adults, and liver tissues of patients with non-malignant hepatopathy.

Enzyme and Isoenzyme

Des-Gamma-Carboxyl Prothrombin (DCP)

DCP is produced by the malignant hepatocyte and appears to result from an acquired post translational defect in the vitamin-K-dependent carboxylase system. DCP production is independent of vitamin K deficiency. DCP levels greater than 100 ng/mL on ELISA are highly suggestive of HCC or tumor recurrence. Normalization of DCP levels correlates well with successful tumor resection and appears to be an excellent marker of tumor activity. It is thought that the combination of AFP and DCP assays will increase the sensitivity of testing.

Gamma-Glutamyl Transferase

Serum gamma-glutamyl transferase (GGT) in healthy adults is mainly secreted by hepatic Kupffer cell and endothelial cell of bile duct and its activity increases in HCC tissue. Sensitivity can be significantly improved with simultaneously determination of GGT, DCP and AFP.

Serum Alpha-1-Fucosidase

Alpha-l-fucosidase (AFU) is a lysosomal enzyme found in all mammalian cells with a function to hydrolyze fucose glycosidic linkages of glycoprotein and glycolipids. Its activity increases in the serum of HCC patients. In combination with AFP it is useful for early diagnosis of HCC.

Human Carbonyl Reductase 2

This enzyme expressed in the human liver and kidney is important in detoxification of the reactive alpha-dicarbonyl compounds and ROS deriving from oxidative stress in HCC. Its levels have been shown to be inversely correlated to the pathological grading of HCC.

Growth Factors and Their Receptors

Transforming Growth Factor-Beta (TGF-β)

TGF-β belong to a superfamily of polypeptide signalling molecules involved in regulating cell growth, differentiation, angiogenesis, invasion, and immune function, it is a predominant form of growth factor family in humans. Its mRNA and protein are overexpressed in HCC compared with surrounding liver tissues, especially in small and well-differentiated HCC.

Tumor Specific Growth Factor (TSGF)

Malignant tumors release tumor-specific growth factor (TSGF) into peripheral blood during their growing period. Serum levels of TSGF may reflect the existence of tumor. TSGF can be used as a diagnostic marker in detecting HCC, and its sensitivity can reach 82% and may have a higher accuracy with the simultaneous determination of other tumor markers. The simultaneous determination of TSGF (at the cut-off value of 65U/mL), AFP (at the cut-off value of 25 ng/mL), and serum ferritin (at the cut-off value of 240 ng/mL) can reach a sensitivity and specificity of 98.4% and 99%, respectively.

Epidermal Growth Factor Receptor Family (EGFR)

The epidermal growth factor receptor (EGFR) family consists of four closely related transmembrane tyrosine kinase receptors, EGFR (erbB-1), c-erb-2 (Her-2/neu), c-erb-3(HER3), and c-erb-4(HER-4). These bind with ligands of the EGF family, including EGF, TGF-alpha, and heparin-binding EGF. High levels of EGFR expression have been associated with early recurrence.

Hepatocyte Growth Factor/Scatter Factor

Hepatocyte growth factor/scatter factor (HGF/SF) is a cytokine with a wide range of effects from embryonic development and liver regeneration.

Table 1: Diagnostic values of HCC serum markers.[24]

Type of test	Specificity (%)	Sensitivity (%)
AFP-L3	92.0	61.6
DCP	90.0	72.7
AFP	71.0	67.7
AFP-L3 + DCP	97.8	84.8
AFP-L3 + AFP	86.6	73.7
DCP + AFP	90.2	84.8
AFP-L3 + DCP + AFP	59.0	85.9

It is associated with molecular mechanisms of hepatocarcinogenesis via paracrine system involving its cellular receptor, c-met. High c-met expression has been shown in invasive-type HCC and has been associated with metastasis and reduced overall survival (Table 1).

MicroRNA

MicroRNAs (miRNAs) are small non-coding RNAs that function as guide molecules in RNA silencing. Targeting most protein-coding transcripts, miRNAs are involved in nearly all developmental and pathological processes in animals. The biogenesis of miRNAs is under

tight temporal and spatial control, and their dysregulation is associated with many human diseases, particularly cancer.[25] MicroRNAs (miRNAs) are endogenous ~23 nucleotide RNAs that play important gene-regulatory roles in animals and plants by pairing to the mRNAs of protein-coding genes to direct their posttranscriptional repression.[26] MicroRNAs have many requisite features of good biomarkers such as: stability in various bodily fluids [27], conserved sequences of most of the miRNAs among different species[28]; tissues or biological stages specific expression[29]; and the level of miRNAs can be easily accessed by various methods, including methods such as RT-PCR, microarray and deep sequencing.[21] MicroRNAs post transcriptionally regulate gene expression by binding to the untranslated regions (UTRs) of target mRNA and exert their influences on biologic processes, such as the proliferation, differentiation, apoptosis, invasion and metastasis.[30] Thus, miRNAs are critical regulators of gene expression, amplification and overexpression of individual 'oncomiRs' or genetic loss of tumour suppressor miRNAs are associated with human cancer and are sufficient to drive tumorigenesis in mouse models.[31]

MicroRNA biogenesis

Process of mature miRNA biogenesis is characterized by three fundamental steps: Cropping of pri-miRNA, export and dicing. MicroRNA (miRNA) genes are evolutionally conserved. Their location is either within the introns or exons of protein-coding genes (about the 70%) or in the intergenic areas (30%) where the expression of the intergenic microRNA is related to their host gene expression, all intragenic microRNA has independent transcription units[32] (Fig. 2).

Cropping

MicroRNAs are transcribed, capped and polyadenylated by RNA polymeraseII. This process generates a long primary transcript called pri-miRNA. The pri-miRNA transcript is several kilobases long and is processed in the nucleus by the Drosha/DGCR8 heterodimer which crops the pri-miRNAs, producing a transcript of about 70 kb, a microRNA precursor called pre-miRNA.

Export

At this point the Exportin 5 (XPO5) and its catalytic partner Ran-GTP bind the pre-miRNA, thereby inducing the export of this molecule from the nucleus into the cytosol.

Dicing

The pre-miRNA is recognized in the cytosol by the RNAseIII Dicer enzyme, which processes the pre-miRNA, producing a mature miRNA duplex of about 22 nucleotides. At this step the miRNA double strand binds to the RNA-induced silencing complex (RISC). RISC is composed of the transactivation-responsive RNA-binding protein (TRBP) and Argonaute2. It retains the mature strand fragment while the complementary strand is removed and degraded resulting in a fully functioning miRNA. microRNAs induce the degradation of their mRNA target through an imperfect complementary matching with the targets 30 UTR. This so called seed region of the microRNAs is very important for their function and target specificity. The ability of even a single miRNA to influence cell identity was further proved in early over expression studies showing that transfecting HeLa cells with a single miRNA, miR-124, shifted the expression profile towards that found in the brain, a tissue in which miR-124 is highly expressed. From their role in differentiation, formation of cellular identity and their noise-dampening effect, it follows that loss of miRNA.

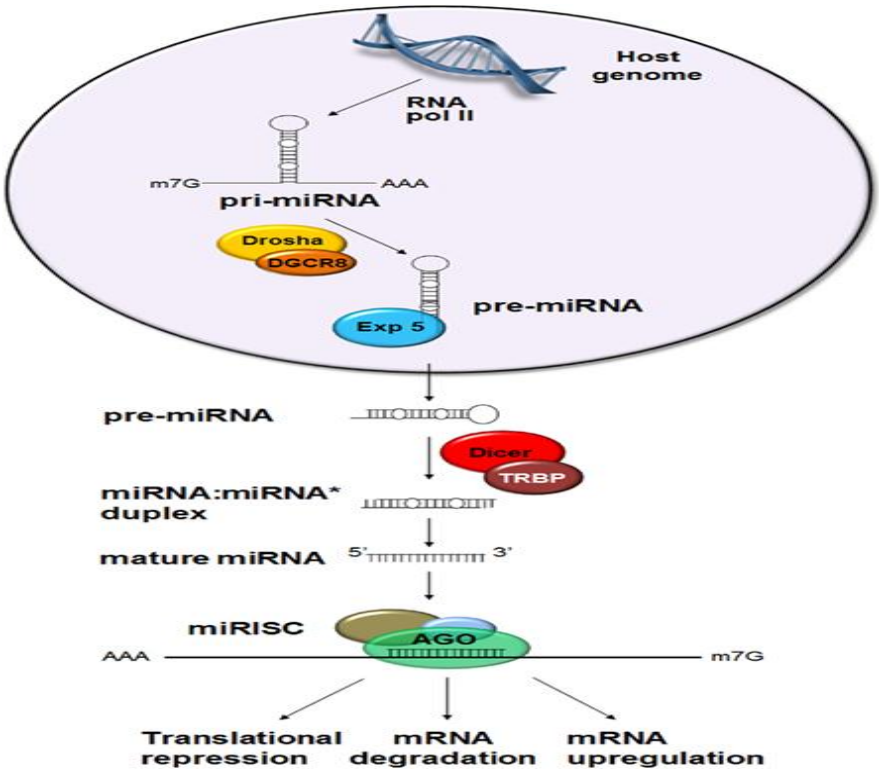

Figure 2: microRNA biogenesis[33]

function may result in increased cellular plasticity, dedifferentiation and a higher propensity for oncogenic transformation.[34] Interestingly, miRNA also play key roles in stem cells and stem cell differentiation.[35] In induced pluripotency where a single miRNA cluster, miR- 302

was recently shown to be able to produce iPSC from both human and mouse fibroblasts.[36] Currently, more than 1400 human miRNAs have been identified many of which are strongly conserved even among distantly related vertebrates and invertebrates. Overall, the potential for miRNA mediated regulation of gene expression is enormous as more than 60% of all mRNAs are predicted to be under miRNA control. Hence, miRNA regulation appears to be the most abundant mode of post transcriptional regulation. The literature on miRNAs and cancer is enormous (>7000) and very diverse both in terms of the diseases in study and the experimental approaches taken. However, Alvearez [37] reported challenge in studying miRNAs, includes.

1. As a particular miRNA targets a host of mRNAs the phenotypic outcome of deregulating even individual miRNAs is unlikely to be mediated via a single target. Although majority of published papers focus on individual targets, most miRNAs like exert their full functional effects via multiple target mRNAs, some of which may reside in the same cellular pathway.
2. Many miRNAs exist in families with similar seed sequences, which complicate the interpretation.
3. Redundancy also exists at the level of the target mRNAs where different miRNAs with distinct seed sequences may repress the same target[38]
4. A particular miRNA may be found up regulated in some cancer types, and thus supposedly oncogenic, but down regulated in other cancers, indicative of tumour suppressor function.
5. Studying miRNA links to cancer is furthermore complicated by the genetic diversity of tumours and cancer cell lines and by that fact that most often many miRNAs are found deregulated in the same tumor.

MicroRNAs as a Biomarker

Molecular classification of HCC

MicroRNAs affected in HCC, and their different dysregulation patterns can be used to discriminate tumors based on molecular characteristics. Toffanin S [39] proposed a miRNA-based classification of HCC in three subclasses: the wingless-type MMTV integration site, Interferon related, and proliferation subclasses. Such miRNA based determination of molecular subclasses of HCC could allow subtype-specific treatment. Thus miRNA may play

a crucial role not only for HCC classification and subtype-specific treatment allocation, but also for prognosis.

As Diagnostic Marker

The lack of effective diagnostic methods for early HCC has rendered the overall survival rate to a low 0-14% from the time of clinical diagnosis. Circulating mi RNAs are highly stable in serum owed to their resistance to RNAse, extreme pH and temperature hence is perfect as biomarker for detecting early stage. The serum levels of miRNAs undergo alterations in HCC patients as evident from low levels of miR-16, miR-199a, and high levels of miR-21, miR-221, miR-222, miR-223, and miR-224 in serum samples. miR-125b is down-regulated in 70% of primary HCC samples thus could be a good candidate for diagnosis. Further to expand the repertoire of prospective miRNAs in early diagnosis of HCC, down-regulated levels of TS-miR-129 in HCC can be detected in plasma samples from85% of stage I, HCC patients as compared to AFP in just 10% of stage I cases. In light of this, unique expression profile of serum miRNAs in HBV and HCV positive HCC patients can serve as a fingerprint for distinguishing between HBV and HCV cases. Not surprisingly, miRNAs such as miR-1269, miR-224, and miR- 224-3p are significantly altered specifically in HCV-associated HCCs. In contrast, miR-152 and miR-143 are aberrantly regulated in HBV-related HCC and hence constitute potential diagnostic markers for HBV related HCC cases. Serum miRNAs enriched in exosomes can also serve as valuable non-invasive HCC biomarkers for both diagnostic and prognostic. Indeed, recent findings have shown that serum exosomal miR-21 from HCC patients provides increased sensitivity of detection compared to whole serum. Interestingly, a report on urinary miRNAs such as miR-618 and miR-650 has opened the prospect to use them as biomarkers for early detection of HCV-induced HCC.[40]

As Prognostic Marker

In addition to their diagnostic potential, miRNAs may be helpful in prediction of the prognosis of HCC.[41] Kaplan-Meier survival analyses revealed an inverse correlation between miR-221 expression and survival rates. In another study[42 it was reported miR-21 expression in plasma from 126 HCC patients as miR-21 expression was high in HCC and diminished after surgical treatment. Most importantly, high miR-21 expression level in plasma correlated with shorter cumulative survival following treatment. In one analysis performed for miR-1 and miR-122 in European HCC patients showed that higher miR-1 and miR-122 serum levels were associated with longer overall survival compared to low

expression of those miRNAs. However, miR-122, but not miR-1, showed a correlation with hepatic inflammation, liver function and synthetic capacity. Thus miR-1 may be a liver function independent predictive biomarker of HCC. There is also growing evidence that miRNA signature profiling can be useful in prognostic stratification. A distinct 20 miRNA signature associated with metastases of HCC has been identified.[43]

miRNAs in HCC Therapy

As one miRNA may target several genes that are involved in the development and maintenance of the HCC phenotype. Therefore, miRNA-based gene therapy offers promising perspectives compared to classical gene therapy for HCC. An additional advantage of miRNAs is that since they encode no protein, they are generally not immunogenic[44]. Alteration in miRNA expression is frequently associated with HCC disease could be used as potential drug target in HCC managements. In terms of therapeutic term antagomirs can be used against oncomiRs. AAV mediated delivery of miR-124 can suppress tumorigenesis in animal model of HCC. Similarly, restoration of miR-375 (2, *O*-methyl-modified and cholesterol conjugated form) and miR-29 could inhibit tumorigenesis in preclinical HCC.

Delivery of miRNA mimics can also be used in HCC therapy. Indeed, cancer targeting miRNA mimic of miR-34 is in clinical trial performed by miRNA therapeutics in HCC patient. Interestingly, the mimic miRNAs are delivered using Smarticles®, which are anionic at neutral pH but attain cationic charge in acidic tumor environment thus minimizing off target effect.[20] Importantly, the therapeutic potential of miR122 antagonist Miravirsen which is in trial showed sequestration of mature miR-122 by inhibiting Dicer and Drosha mediated processing of miR-122 and reduce viral load.[45] A major concern remains their delivery system miRNA can be incorporated to PEGylated stable nucleic acid lipid particles (SNALPS) to extend the circulation time.

The emergence of miRNAs as novel clinical biomarkers is set to change the face of HCC diagnosis and therapeutic procedure. The proposition of miRNA profiles serving as signatures to distinguish HCV from HCB cases though awaiting clinical evaluation offers the advantage of accurate diagnosis and appropriate therapeutic course [46] (Table 2).

Table 2: Circulating microRNA candidate biomarker of HCC[44]

miRNA	Dysregulation in plasma	Experimental setting	Result
miR-16	Down	71 HL, 105 HCC, 107 CLD	Significance association with HCC, combination with traditional maker improve diagnostic
miR-21	Up	86 HL, 101 HCC, 48 CH	Elevated in HCC
miR-122	Up	89 HL, 101 HCC, 48 CH	Unspecific maker of liver injury
miR195	Down	71 HL, 105 HCC, 107 CLD	Significant association with HCC
miR-224	Up	86 HL, 101 HCC, 48 CH	Elevated in HCC
miR-885	Up	10 HL, 15 HCC, 10 LC	Marker of liver cirrhosis

MicroRNA expression profile in HCC

Oncogenic miRNA in HCC

There are some reported microRNAs which are highly up regulated in HCC, like miR-221/222, miR-21, miR-224 and miR-34a[47] (Table 3).

miR-221/222

The study *in vitro* and *in vivo* analysis reported miR-221 as oncogenic that accounts for 71% of cirrhotic tissue HCC by increasing cellular proliferation and migration. miR-222 and miR-221 are homologous miRNAs that function similarly in the pathogenesis of HCC. miR-221 and miR-222 also induce tumor necrosis factor-related apoptosis inducing ligand resistance and enhance cellular migration by modulating the expression of phosphatase and tensin homolog (PTEN) and TIMP3. In addition pro-apoptotic protein B-cell lymphoma 2-modifying factor (BMF) and the DNA damage-inducible transcript 4, have been validated as targets of miR-22. Therefore, miR-221 exerts its tumor-promoting function by regulating certain downstream genes that are involved in cancer-related processes.[48]

miR-224

The expression of miR-224 is undetectable in normal liver tissues, however as liver disease progresses, the levels of miR-224 increase therefore it is consider as a hallmark of HCC. Furthermore miR-224 reported as part of the lipopolysaccharide, lymphotoxin α and tumor necrosis factor α (TNFα) inflammatory pathways, and target HOXD10 thus act as a link between cell migration and invasion in HCC.[49] Therefore, miR-224 miRNA has multiple roles in the pathogenesis of liver cancer, and appears to be a useful biomarker for clinical diagnosis.

miR-21

It has been reported that miR-21 regulate MAP2K3 in HCC by down regulating MAP2K3 in HCC tissue, which further validated as novel target.[50] the silencing of PTEN, an important tumor suppressor, miR-21 promotes tumor cell proliferation, migration and invasion in HCC. miR-21 lowered programmed cell death 4 (PCD4) protein expressions in HBV-related HCC, which weakened the tumor suppressive effects of PCD4 in liver cancer. In addition, mitogen-activated protein kinase-kinase 3 (MAP2K3) has been confirmed as one of the downstream targets of miR-21 that is involved in liver tumor cell proliferation.[51]

Tumor suppressive miRNAs in HCC

Certain subsets of miRNAs are reported to be silenced in human liver cancers, and therefore function as tumor suppressors. Screening from miRNA profiling most frequently identifies miR-122, miR-125a/b, miR-26, miR-199 and miR-375 as tumor suppressive miRNAs.[22, 48]

miR-122

It constitutes 70% of the total adult liver miRNA content, target many genes that are involved in regulation of tumorrigenesis and cancermetastasis. These include Pkm2, HNF4A, RHOA, VEGF, vementin, a disintegrin and metalloprotease, serum response factor, insulin-like growth factor 1 receptor and ADAM17. Therefore, miR-122 has a central role in the suppression of HCC.[48]

Table 3: Major aberrantly expressed miRNA and target genes in HCC.[48]

miRNA	Targets
Down regulated	
miR-122	Pkm2, HNF3B, RHOA, ADAM10, ADAM17, Cyclin G1, IGF1R, Bcl-w
miR-26	Lin28B, Zcchc11, Era, cyclin D2, cyclin E2, MTDH, EZH2, NF-κB pathway
miR-199a	Kras, TIMP3, Fibronectin
Upregulated	
miR-221	p27, p57, Bmf, PTEN, TIMP3, DDIT4
miR-222	p27, PTEN, TIMP3, PPP2R2A
miR-21	MAP2K3, PTEN, RhoB
miR-224	Smad4, HOXD10, API-5, NF-κB pathway

CONCLUSION

Research into the molecular biology of hepatocarcinogenesis has identified numerous biomarkers which could provide additional information for HCC biologic behavior metastasis and recurrence of HCC. However non-invasive and more sensitive molecular biomarkers

remain needed to complete and improve the current strategies for detection. Specificity along with remarkable stability make serum microRNA a useful biomarker. Profiling miRNAs in the serum might be used to improve disease diagnosis by distinguishing healthy from malignant tissues, identifying the tissue of origin in poorly differentiated tumors or tumors of unknown origin, and distinguishing the different subtypes of the same tumor. Additionally, serum miRNA profiling could be used for cancer classification, prognosis estimation, prediction of therapeutic efficacy, maintenance of surveillance. The authors report no conflict of interest in this work.

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