Validation of mRNAs in Early Diagnosis and Treatment of Hepatocellular Carcinoma

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ABSTRACT

Poor and late diagnosis of HCV is main the cause of liver cancer. MicroRNAs are non-coding molecules that are involved in regulation of a variety of functions happening in the cell, in healthy and diseased state. Dysregulation of microRNAs is observed in different diseases, especially in liver cancer like hepatocellular carcinoma. The available detection methods detect HCC at a late stage. There is a need to find novel biomarkers for diagnosis at an earlier stage to minimize chances of liver cancer. Circulating microRNAs are novel and minimal invasive markers for early detection of HCV based hepatocellular carcinoma. In this review, the current progress on the potential role of miRNA as biomarkers for detection of HCC and therapeutic targets are summarized. We concluded that the expression of microRNAis upregulated in the patients of hepatocellular carcinoma when compared with the healthy ones. In-depth studies of miRNA in patients of HCC as genetic biomarkers will improve the diagnosis. It will also improve the prognosis of early stage HCC patients. This will also help in identifying a suitable and effective therapeutic targets so as to reduce the chances of failure of chemotherapy.

Keywords: Hepatocellular Carcinoma (HCC), microRNAs, mRNA, Biomarkers for HCC, MicroRNA Biogenesis

Introduction

Thousands of patients infected by HCV develop persistent and chronic hepatitis which usually out-turn in liver cirrhosis and sometimes even terminate to hepatocellular carcinoma. According to the estimate of the World Health Organization (WHO), about 3% of the world population is infected by HCV. Chronic HCV is among primary risk factors of hepatocellular carcinoma [1]. In terms of number of cases, hepatocellular carcinoma (HCC) is sixth most common cancer worldwide and the second main contributor to cancer mortality in man [2]Esat Asia and Africa are regions with highest burden of HCC but there is a rapid rise in disease incidence and mortality in the United States and Europe [2]. HCC may be fatal mainly due to late or poor diagnosis. Many methods are used for its diagnosis, but they are not so sensitive and accurate. These methods include Magnetic Reasoning Imaging (MRI), Spiral Computed Tomography (CT), Ultrasound Scan (US) and Alpha Fetoprotein (AFP), [3]. CT and MRI can provide images of high-resolution than the US, but they are more costly, and CT is also associated with radiation exposure [4]There are some tumor markers for HCC that have been reported to have prognostic significance. HCC progression is reflected by the elevation of these markers, but prognostic significance of these tumor markers is lower in patients with early stage [5].

Circulating microRNAs are novel and minimal invasive markers for early-stage detection of HCV based hepatocellular carcinoma. In this review, the current progress on the potential role of miRNA as biomarkers for detection of HCC and therapeutic targets for HCC treatment are summarized. The first section deals with miRNAs and their biogenesis. In the second section their role in HCC has been elaborated.

MicroRNA

MicroRNAs are small non-coding RNAs with the length of 19-24 nucleotides [6]. In many organisms microRNA acts as a guide molecule for post-transcriptional gene regulation. These microRNAs are part of many biological processes which include development, proliferation of cells, metabolism and signal transduction. Recent studies have revealed that microRNAs are present in significant amounts in different kinds of fluids in the body such as blood serum and

blood plasma. These biomolecules can withstand harsh physiological conditions like multiple freeze-thaw cycles and severe changes in heat and pH [7].The sequences of many miRNAs are conserved between distantly related organisms. This suggests the crucial role of molecules in vital processes [8]. Each miRNA has hundreds or thousands of targets, and a significant part of the mammalian transcriptome is regulated by these small molecules [9]. miRNAs play an essential role in regulation of genetic programs and developmental pathways. In this way, they exert influence to strengthen or alter the molecular pathways susceptible to variations in genetic expression. The expression of miRNAs has been reported in many types of physiological processes and multiple pathways. This include pancreatic cell insulin secretion (*miR-375*), brain patterning (*miR-430*), and adipocyte development (miR-145), B-cell lineage fate (miR-181, cell proliferation control (miR-125b and let-7) and B-cell survival (miR-15a and miR-16-1) Besides this, a large number of studies have reported its role in prognosis and progression of many diseases[8].Two small non-coding sequences of 22 and 61 nucleotides of lin-4 were identified with the identification of seven elements in 3' untranslated region (UTR) of lin-14 that had complimentary sequence to the lin-4 small RNAs in independent studies [10].

MicroRNA Biogenesis

MicroRNAs are translated by RNA polymerase II as primary microRNA that is several kb in length. This product is known as primary- microRNA. These RNAs are usually several kilo bases in length. Nearly half of these primary microRNAs are non-coding RNAs as significant open reading frames are not present. These primary microRNAs are clustered in such a way that single pri-microRNA contains multiple microRNAs. MicroRNA is contained within a sequence of approximately 60-80 nucleotides, which form a stem-loop hairpin structure by folding back on itself. A microprocessor complex is formed by RNase III enzyme Drosha and its binding partner DGCR8, that recognize and cut primary-microRNA [11].

These processed microRNAs called as pre-microRNA are exported to the cytoplasm by nuclear export factor exportin 5 [12]. In cytoplasm, another RNase III enzyme, Dicer produces an approximately 18-24 nucleotide duplex. Now the fully refined duplex is integrated into a large protein complex RISC (RNA-induced silencing complex); this process is ATP –independent. One strand of microRNA that remains attached with RISC becomes the mature microRNA. The other strand is disposed of through some alternative mechanism [12].

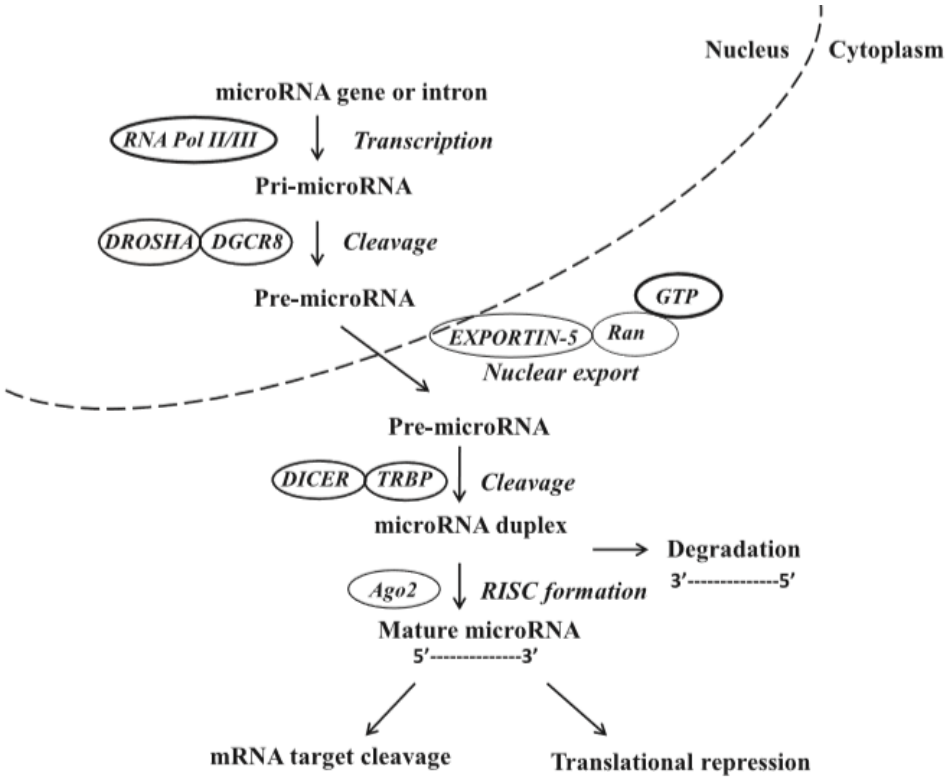

Fig 1. A schematic representation of miRNA biogenesis pathway. In the nucleus, the pri-miRNA transcribed by RNA pol II or III and cleave by the Drosha-DGC8 complex. The yielded hairpin precursor or pre-miRNA is transported from nucleus to cytoplasm via the exportin-5-Ran GTP complex. The RNAse Dicer combine with TRBP to form a complex and cleaves the pre-miRNA hairpin to a miRNA duplex. This mature miRNA along with Agronaute (Ago2) proteins and RISC targets the mRNAs by cleavage and deadenylation. The passenger strand is then degraded [13].

MicroRNAs in Carcinomas

The complete complement of microRNAs which are present in a genome are referred to as microRNoma . The changes in expression of microRNoma in tumor cells is signified by

abnormal expression level for mature miRNA and precursor miRNA sequences when compared with the normal tissue [9]. Involvement of microRNA in cancer was discovered by Carlo and Celin. Deregulation of microRNA was found in the cancerous tissues. In human tumors the expression of microRNA may be widely up regulated or down regulated, relative to normal tissues [14]. There are numerous hallmarks of tumerogenesis which are believed to be regulated by miRNAs. This includes abnormal apoptosis, replicative immortality, insensitivity of antigrowth signals, evocation of angiogenesis and metastasis [8].

Dysregulated miRNAs expression has been studied in various tumors, lung, prostate, colon, breast and also liver cancers [5].It has been shown to alter the regulatory activity of the oncogenes and tumor suppressor genes, which is, thereby directly affecting carcinogenesis [15].

MicroRNA binding sites were reported in the coding sequence of genes Sox2, Oct4 and Nanog in mouse, where microRNA bind and adjust cell differentiation in mouse. Sensitive PCR techniques can detect the level of microRNAs in serum. After some studies [16]. Another study a reportsabout detection of placental microRNAs in maternal plasma [16]. Similarly, in all types of cancers the deregulation of microRNA can be used for early detection. A remarkable finding was, If systematically administrated, MicroRNAs can act as anti-cancer therapy [17].

The mechanism for the functioning of microRNA was reported by [18].It was found that microRNA is involved in gene silencing. Additionally a new concept of molecular decoys was introduced, according to this microRNAs work as molecular decoys for regulatory and RNA-binding proteins. It was revealed by a study that over expression of miRNA-21 is a cause of tumor formation in mice [19].

By the year 2000, discovery of two members of small non-coding RNAs family by Ambros and Ruvkun laboratories were done. These were found in nematodes, plants as well as in mammals. In further studies microRNA's regulatory functions in eukaryotes were discovered in next years. Drosha and Dicer are involved in miRNA biogenesis pathway and are present in nucleus and cytoplasm respectively [20]

Role of MicroRNA in Hepatocellular Carcinoma

. A significant role for miRNAs in the progression of cancer has evolved over the past decades. Hepatocellular Carcinoma is a type of malignant tumor in liver. It is one of the most common tumors and is on third number in death due to cancer [21]. The cause of this tumor may be viral (HBV, HCV) or non-viral (Aflatoxin B1). It is reported that chronic hepatitis C viral infection can be a leading cause of hepatocellular carcinoma. Generally, HCC may develop just after one or two decades of hepatitis C infection. [22]. Patients with cirrhosis or advanced stage of fibrosis are more likely to be at risk of developing HCC disease. Successful antiviral therapies of HCV patients may decrease the risk of hepatocellular carcinoma [23].

It is observed that different types of circulating microRNAs are correlated with manifestation, invasion and metastasis of cancer. This discovery suggests the role of microRNA as a diagnostic tool for the detection of cancer. Tumor formation and cell cycle dysregulation are also related to miRNA. In tumor cells, changes in miRNA's target binding sites and processing machinery are the key factors which demonstrate its importance in cancer studies. In addition, to distinguish normal and cancer tissues, their distinct miRNA signature is the distinguishing factor. Location of most of the microRNAs are the sites in the human genome that are amplified, deleted or rearranged in cancer, very frequently. This clearly shows the role of microRNA abnormalities in cancer pathogenesis [8].Expression of microRNA may be up or down regulated in HCC patients. Expression of MiR-222 is up-regulated in HCC patients. MiR-122 is down regulated in HCC patient [7].

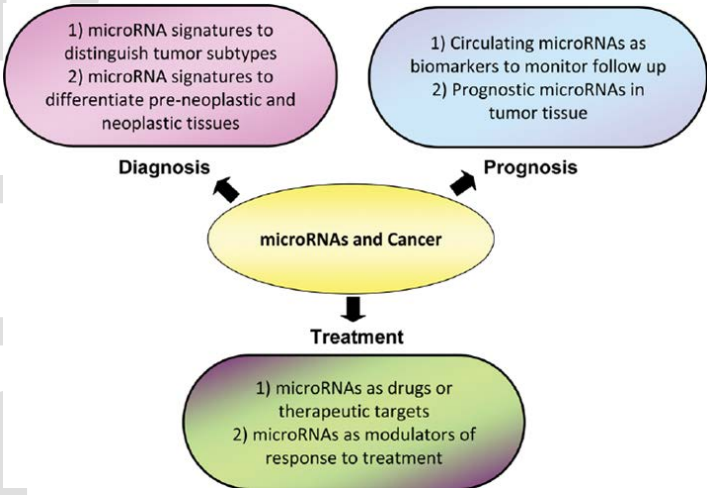

Figure 1: miRNAs used in the Prognosis, Diagnosis and Treatment of Cancer [24].

MicroRNA as biomarker for HCC

Hepatocellular Carcinoma is a complex disease, which can be defined by sequential accumulation of genetic and epigenetic changes. The frequency of HCC is highest in those areas where hepatitis B virus is endemic such as in East Asia. There are multiple risk factors that may concern with this disease; these include chronic infection of HBV/HCV, gender, age, consumption of alcohol and Aflatoxin B exposure [25].

A recent study has revealed that there is some kind of dysregulation in the expression of microRNA during the development and progression of cancer. Detection of microRNAs can be done by several different methods, which include microarrays, bead-based arrays and quantitative real-time PCR [26].

For the differentiation between cancerous and non-cancerous liver tissues, a profile of 69 miRNAs is involved. Eight of these were observed for further distinguishing between benign and malignant tumors [27].

Hepatocellular carcinoma is a type of primary liver cancer. HCC is the third leading cause of death and thought to be fifth prevalent cancer. Of all primary liver cancers HCC accounts for 85-90% [28].

Incidence of HCC is a complex interaction between genetic and non-genetic host factors, exposure to environmental carcinogens and viruses, progression of some chronic liver disease leading to cirrhosis that is a platform for HCC. The liver is a rare target of standard cancer predisposition syndrome. HCC is an exceptional case that can develop in patients with germ-line mutations. In

several genetic metabolic diseases HCC predispositions have been observed, mainly through development of cirrhosis [29].

During progression of HCC dysregulation of genes occur, these are involved in biological processes i.e. cell cycle, growth of cell, cell migration and spreading, this deregulation may be due to the exposure to hepatotoxic agents. During past few decades the focal point of studies is the investigation of those proteins and genes that are involved in the progression and development of HCC [30].

In spite of great advances in the disease treatment, survival rate for HCC is very low. The progression and development of HCC is a multistage process. There are many factors that can trigger the stimulus for hepatocellular carcinoma, such as HBV or HCV infection, intake of Aflatoxin B1 [31].

The diagnosis of HCC is dependent on early detection. It is recommended by AASLD (American Association of the Study of Liver Diseases) that patients who are at high risk of developing HCC must undergo formal surveillance, while periodically being screened for HCC. The objective is the detection of HCC at early stages so that treatments can be applied, which include transplantation of liver and surgical resection. The strategy for detection of HCC includes the use of an ultrasound every 6 months for the detection of any abnormality in liver. At present only two serological assays, alpha-fetoprotien test (AFP) and DCP test are approved for HCC diagnosis by FDA [32].

Many studies are showing a significant relationship between HCC progression and a new class of regulatory RNA molecules known as microRNA. First report was published in 2002, which identified the relationship between microRNA and cancer. After this report, different laboratories started working on it and identified unusual expression of microRNAs in different types of malignancies, for example, breast cancer, lung cancer, brain cancer, hepatocellular carcinoma [33].

In different carcinogenic processes including metastasis, invasion, proliferation, cell cycle, and apoptosis, the role of microRNA is varied. Mapping of microRNA revealed that many of them are present on delicate regions of the genome and they have decreased expression in cancer cells. MicroRNA exhibit dual roles including both oncogenic and tumor suppressive and also participate in normal cellular processes. In normal and tumor conditions microRNAs show differential expression, this difference shows its role as prognostic markers in cancer patients [34].

Since the discovery of microRNAs it is demonstrated that these small, non-coding RNAs are a prevalent class of regulatory RNAs, and are involved in many biochemical mechanisms through their function of gene regulation. But their role in regulation of gene expression is still unclear. The microRNA degrades the mRNA or blocks the translation of mRNA by binding itself to the 3' UTR of mRNA. For better understanding of the function of microRNA, identification of its target is an important factor [35]. In the meantime, deregulation of microRNA was observed in a large number of diseases, including cancer [36]. MicroRNA may also perform the function of tumor suppressor genes during tumor development in human cancer [37].

In the progression and development of human cancer, defects in the cell cycle are an important step. In the regulation of cell cycle there are a lot of oncoproteins and tumor suppressors involved, these tumor suppressors fail to perform their function in HCC patients leading to cell proliferation. Studies have revealed that microRNA can interact with some cell cycle regulators, for example cyclin-cyclin dependent kinase enzyme complexes (CDK), through this interaction microRNA can regulate cell proliferation pathways [38].

Tumor cells escape from the surveillance system of cells due to some evolutionary factors that help them to evade apoptosis. Apoptosis is partially seized during tumor progression. By targeting related apoptotic genes, microRNA can regulate apoptotic cell death. In HCC patients, miR-224 is upregulated and inhibits the inhibitor for apoptosis thus increasing cell proliferation [39].

For malignant cancer, invasion and metastasis are two leading lethal factors. High recurrence rate of HCC is a major complication which is mainly due to intrahepatic metastasis spread, and is overwhelmed after long term survival of its patients after medicinal resection. For the treatment of HCC, understanding the mechanisms of metastasis and identification of metastatic factors are important. Metastasis related genes are regulated by a number of upstream regulators i.e. pro-metastatic miRNAs and anti-metastatic miRNAs. These regulators have a significant role in the progression and metastasis of HCC cells [40].

Several studies revealed that expression profiles of miRNA have signatures for classification of tumor, diagnosis and disease progression. As a single microRNA can control several mRNAs so

if a single microRNA is disturbed, it can affect the expression of several mRNAs and proteins. MicroRNA expression profiles can detect the tissue of origin of cancer [41].

Developmental lineage and differentiation state of tumor was demonstrated by the expression analysis of 217 microRNAs in different human cancers. It was observed that microRNA down regulates in the cancerous tissues as compared to the normal tissues. Several studies showed the usefulness of circulating microRNAs as diagnostics and prognostics. This reveals the importance of microRNA profiling in cancer diagnosis [42]. Table 1 presents a summary of different types of cancer and mRNA Biomarkers reported so far.

Table 1: Examples of cancer and corresponding published miRNA biomarkers

Type of Cancer	Biomarker	Reference
Diffuse large B-cell lymphoma (DLBCL)	Expression level of miR-155, miR-210 and miR-21 was high in DLBCL patients.	[43]
Gastric Cancer	The expression of miR-17-5p, miR-21, miR-106a, and miR-106b was significantly higher in plasma, whereas let-7 showed down regulated expression.	[44]
Pancreatic Cancer	Elevated levels of Circulating miR-210 in patients	[45]
Squamous cell carcinoma (SCC) of tongue	MicroiRNA-184 levels were significantly higher in plasma and the levels were significantly reduced after surgical removal of the primary tumors	[46]
Hepatocellular Carcinoma	Increased MiR-500 level was found in the sera of the HCC patients.	[47]

Using a global miRNA expression profile in mouse liver development it was observed that miR-500 is an oncofetal miRNA in liver cancer. Its expression was high in the fetal liver. Its expression was down-regulated in the developmental process and during liver cirrhosis it was up-regulated. It was reported that microRNA-500 was highly expressed in human cancer cell lines and around 45% of human hepatocellular carcinoma tissues. Presence of abundant amounts of microRNA-500 in circulating blood suggests its importance as a novel diagnostic biomarker. Abundance of circulating microRNA-500 in serum of HCC patients may reflect pathological conditions [47].

Hepatocellular tumors can be classified according to pathological, clinical, and genetic features by microRNA profiling. MicroRNAs are deregulated in tumors as compared to the non-tumor liver samples. MicroRNA-224 was significantly up-regulated in both malignant and benign tumors, while miR-422b and miR-122a down-regulated in both types of tumors. There are some microRNAs that show different behavior in both benign and malignant tumors. MiR-200c and miR-203 were down-regulated in benign tumors, while miR-224, miR-21, miR-222, miR-10b were up-regulated in Hepatocellular carcinoma [34].

Overall, circulating miRNAs are shown to be promising tools to diagnose early stage HCC. Further research should be carried out with larger cohorts to evaluate the diagnostic performance of the different sets of miRNAs [15].

There may be some complications in developing effective biomarkers for HCC due to etiology-related differences. In a current study, for the identification of HBV-HCC or HCV-HCC associated microRNAs, microRNA expression profiling was used [48].

Table2: Differential expression of mRNAs corresponding to etiology related HCC in published literature

MicroRNA	**HCC Etiology**	**Differential Expression**	**Reference**
Let-7a	HBV associated	Down-regulated	[49]
MiR-22	HBV associated	Down-regulated	[50]
MiR-29c	HBV associated	Down-regulated	[51]
miR-99a	HBV associated	Down-regulated	[51]
miR-101	HBV associated	Down-regulated	[51]
miR-150	HCV associated	Down-regulated	[52]
miR-146	HCV associated	Down-regulated	[49]
miR-145	HCV associated	Down-regulated	[53]
miR-142	HCV associated	Down-regulated	[49]
miR-141	HCV associated	Down-regulated	[49]
miR-136	HCV associated	Down-regulated	[49]

miR-139	HCV associated	Down-regulated	[49]
	-------	Down-regulated	[54]
miR-21 38. 110. 113	HCV and HBV associated	Up-regulated	[52]
miR-107	HCV and HBV associated	Up-regulated	[56]
miR-135a	HCV and HBV associated	Up-regulated	[52]
miR-222	HCV associated	Up-regulated	[57]
miR-221	HCV associated	Up-regulated	[52]
miR-224	HCV associated	Up-regulated	[58]

Conclusion

We concluded that the expression of microRNA is upregulated in the patients of hepatocellular carcinoma when compare with the healthy ones. In-depth studies of miRNA in patients of HCC as genetic biomarkers will improve the diagnosis. It will also improve the prognosis of disease at early stages. This will also help in identifying suitable and effective therapeutic targets so as to reduce the chances of failure of chemotherapy.

Conflict of Interest

The authors declare no conflict of interest.

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