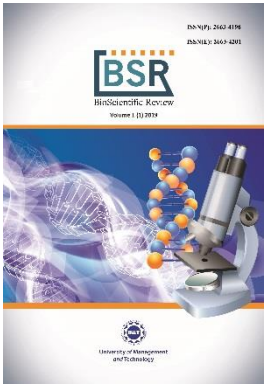

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Validation of MicroRNAs (miRNAs) in the Early Diagnosis and Treatment of Hepatocellular Carcinoma (HCC)

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Abstract

Late diagnosis and delayed presentation of HCV is among the leading causes of liver cancer. MicroRNAs (miRNAs) are non-coding molecules involved in the regulation of a variety of functions happening in the cell, in both healthy and diseased states. Dysregulation of miRNAs is observed in different diseases, especially in liver cancer including hepatocellular carcinoma (HCC). The available detection methods detect HCC at a late stage. There is a need to find novel biomarkers for the diagnosis of HCC at an early stage in order to minimize the chances of liver cancer. Circulating miRNAs are novel and minimally invasive markers used for the early detection of HCV based HCC. In this review, the current progress on the potential role of miRNAs as biomarkers used for the detection of HCC and other therapeutic targets was summarized. We concluded that the expression of miRNAs is upregulated in the patients of HCC as compared with healthy individuals. An in-depth study of miRNAs as genetic biomarkers in the patients of HCC will improve its diagnosis. It will also improve the prognosis of early stage HCC patients. Furthermore, it will also help to identify a suitable and effective therapeutic target so as to reduce the chances of the failure of chemotherapy.

1. Introduction

Thousands of patients infected by HCV develop persistent and chronic hepatitis which usually out-turns in liver cirrhosis, and sometimes, even leads to hepatocellular carcinoma (HCC). According to the estimate of the World Health Organization (WHO), about 3% of the world population is infected by HCV. Chronic HCV is counted among the primary risk factors of HCC [1]. In terms of the number of cases, HCC is the sixth most common cancer worldwide and the fourth leading cause of mortality due to cancer [2]. East Asia and Africa are regions with the highest burden of HCC, although there has been recorded a rapid rise in disease incidence and mortality in the United States and Europe also [2]. HCC is fatal mainly due to late or poor diagnosis. Many methods are used for its diagnosis; however, 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 more high-resolution images than the ultrasound scan; however, they are more costly and CT is also associated with radiation exposure [4]. There are some tumor markers for HCC that reportedly have prognostic significance. HCC progression is reflected by the elevation of these markers, although prognostic significance of these tumor markers is lower in patients with disease at an early stage [5].

Circulating microRNAs (miRNAs) are novel and minimally invasive markers for early stage detection of HCV based HCC. In this review, the current progress on the potential role of miRNAs as biomarkers used for the detection of HCC and also as therapeutic targets for HCC treatment are summarized. The first section deals with miRNAs and their biogenesis. In the second section, their role in HCC detection is elaborated.

2. MicroRNAs (MiRNAs)

MiRNAs are small non-coding RNAs with a length of 19-24 nucleotides [6]. In many organisms, miRNAs act as guide molecules for post-transcriptional gene regulation. MiRNAs are part of many biological processes including the development and proliferation of cells, as well as metabolism and signal transduction. Recent studies have revealed that miRNAs are present in significant amounts in different kinds of fluids produced by the body, such as blood serum and blood plasma. These biomolecules can withstand harsh physiological conditions such as multiple freeze-thaw cycles and severe changes in heat and pH [7]. The sequences of many miRNAs are conserved between distantly related species. This suggests the crucial role of these molecules in vital life 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]. They play an essential role in the 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. These include pancreatic cell insulin secretion (*miR-375*), brain patterning (*miR-430*), adipocyte development (miR-145), B-cell lineage fate (miR-181), cell proliferation and control (miR-125b and let-7) and B-cell survival (miR-15a and miR-16-1). Besides these, a large number of studies have reported its role in the prognosis and progression of many diseases [8]. Two small non-coding sequences of 22 and 61 nucleotides of lin-4 were identified along with seven elements in the 3' untranslated region (UTR) of lin-14. They showed a complimentary sequence to the lin-4 small RNAs in independent studies [10].

2.1. MicroRNA Biogenesis

MiRNAs are translated by RNA polymerase II and III. This product is known as the primary miRNA. These RNAs are usually several kilo bases in length. Nearly half of these primary miRNAs are categorized as non-coding RNAs, since significant open reading frames are not present. These primary miRNAs are clustered in such a way that a single primary-miRNA contains multiple miRNAs. MiRNAs are contained in a

sequence of approximately 60-80 nucleotides, which forms 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, which recognize and cut primary-miRNA [11].

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

Figure 1. A schematic representation of miRNA biogenesis pathway. In the nucleus, the pri-miRNA is transcribed by RNA pol II or III and cleaved by the Drosha-DGC8 complex. The yielded hairpin precursor or pre-miRNA is transported from the nucleus to cytoplasm via the exportin-5-Ran GTP complex. The RNAse Dicer combines with TRBP to form a complex and cleaves the premiRNA 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].

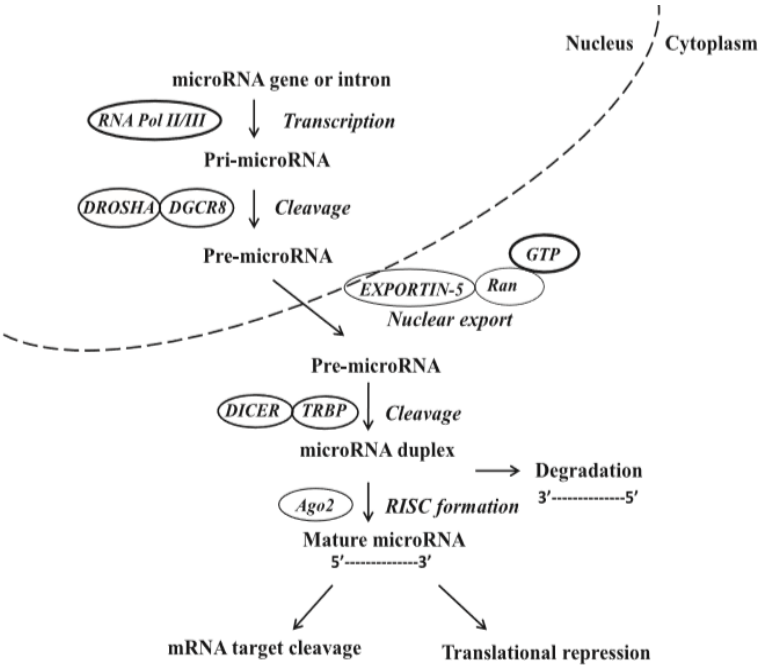

Figure 1. miRNAs used in the prognosis, diagnosis and treatment of cancer [24]

3. MicroRNAs in Carcinomas

The complete complement of miRNAs present in a genome are referred to as microRNoma. Changes in the expression of microRNoma in tumor cells are signified by an abnormal expression level for mature miRNA and precursor miRNA sequences, as compared with the normal tissue [9]. The involvement of miRNAs in cancer was discovered by Carlo and Celin. It was found that miRNAs were deregulated in cancerous tissues. In human tumors, the expression of miRNAs may be widely upregulated or downregulated, relative to normal tissues [14]. There are numerous hallmarks of tumerogenesis which are believed to be regulated by miRNAs. These include abnormal apoptosis, replicative immortality, insensitivity of antigrowth signals, evocation of angiogenesis and metastasis [8].

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

A study reported the detection of placental miRNAs in maternal plasma [16]. Similarly, in all types of cancers, the deregulation of miRNAs can be used for early detection. A remarkable finding was that, if systematically administrated, miRNAs can act as an anti-cancer therapy [17].

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

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

3.1. Role of MiRNAs in HCC

A significant role for miRNAs in the progression of cancer has been explicated over the past decades. HCC is a type of malignant tumor in liver. It is one of the most common tumors and ranks third among cancers based on the numbers of deaths caused by it [21]. The cause of this tumor may be viral (HBV, HCV) or non-viral (Aflatoxin B1). It has been reported in previous studies that chronic HCV can be a leading cause of HCC. Generally, HCC may develop after one or two decades of developing HCV [22]. Patients with cirrhosis or advanced stage of fibrosis are more likely to be at risk of developing HCC. However, successful antiviral therapies of HCV patients may decrease the risk of HCC [23].

It has been observed that different types of circulating miRNAs are correlated with the manifestation, invasion and metastasis of cancer. This observation suggests the use of miRNAs as a diagnostic tool for the detection of cancer. Tumor formation and

cell cycle dysregulation are also related to miRNAs. In tumor cells, changes in miRNAs' target binding sites and processing machinery are the key factors which demonstrate their importance in cancer studies. Moreover, to distinguish normal and cancer tissues, their distinct miRNA signature is used. The location of most miRNAs are the sites in human genome that are amplified, deleted or rearranged in cancer, very frequently. This clearly shows the role of miRNA abnormalities in cancer pathogenesis [8]. The expression of miRNAs may be up or downregulated in HCC patients. The expression of MiR-222 is upregulated in HCC patients, while the expression of MiR-122 is downregulated in such patients [7].

3.2. MicroRNAs as Biomarkers for HCC

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

A recent study found dysregulation in the expression of miRNAs during the development and progression of cancer. The detection of miRNAs is possible 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 used. Eight of these were used in this research for further distinguishing between benign and malignant tumors [27].

The incidence of HCC comprises a complex interaction between genetic and non-genetic host factors, exposure to environmental carcinogens and viruses, and the progression of any chronic liver disease leading to cirrhosis that becomes 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 the development of cirrhosis [28, 29].

During the progression of HCC, dysregulation of genes occurs. These genes are involved in various biological processes, such as cell cycle, growth of cell, as well as cell migration and spreading. This dysregulation may be due to the exposure to hepatotoxic agents. During the past few decades, the focal point of studies was the investigation of the proteins and genes involved in the progression and development of HCC [30].

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

The diagnosis of HCC depends 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 (including periodically

getting screened for HCC). The objective is the detection of HCC at an early stage so that different treatments can be applied, which include the transplantation of liver and surgical resection. The strategy used for the detection of HCC includes the use of an ultrasound every 6 months for the detection of any abnormality in the liver. At present, only two serological assays, namely, Alpha-fetoprotien Test (AFP) and DCP test have been approved for HCC diagnosis by FDA [32].

Many studies showed a significant relationship between HCC progression and a new class of regulatory RNA molecules known as miRNAs. The first such report was published in 2002. It identified the relationship between miRNAs and cancer. After this report, different laboratories identified the unusual expression of miRNAs in different types of malignancies, for example, breast cancer, lung cancer, brain cancer, and HCC [33].

In different carcinogenic processes including metastasis, invasion, proliferation, cell cycle, and apoptosis, the role of miRNAs is varied. The mapping of miRNAs revealed that many of them are present in delicate regions of the genome and they have decreased expression in cancer cells. MiRNAs exhibit dual roles including both oncogenic and tumor suppressive roles and also participate in normal cellular processes. In normal and tumorous conditions, miRNAs exhibit a differential expression which shows their role as prognostic markers in cancer patients [34].

Since the discovery of miRNAs, it has been demonstrated that these small, non-coding RNAs comprise a prevalent class of regulatory RNAs. They are involved in many biochemical mechanisms through their function of gene regulation. However, their role in the regulation of gene expression is still unclear. They degrade the mRNA or block the translation of mRNA by binding itself with the 3' UTR of mRNA. For better understanding of the function of miRNAs, the identification of their targets is an important factor [35]. Furthermore, the deregulation of miRNAs has been observed in a large number of diseases, including cancer [36]. MiRNAs may also perform the function of tumor suppressor genes during tumor development in 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 miRNAs can interact with some cell cycle regulators, for example, Cyclin-Cyclin Dependent Kinase (CDK) enzyme complexes. Through their interaction, miRNAs 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, miRNAs 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.

The high recurrence rate of HCC is a major complication which mainly arises due to intrahepatic metastasis spread and is overwhelmed after the long-term survival of its patients after medicinal resection. For the treatment of HCC, understanding the mechanisms of metastasis and the identification of metastatic factors is important. Metastasis related genes are regulated by a number of upstream regulators, that is, 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 the expression profiles of miRNAs have signatures for the classification of tumor, their diagnosis and disease progression. A single miRNA can control several mRNAs. So, if a single miRNA is disturbed, it can affect the expression of several mRNAs and proteins. MiRNA expression profiles can detect the tissue-of-origin of carcinoma [41].

Developmental lineage and differentiation state of tumors were demonstrated by the expression analysis of 217 miRNAs in different human cancers. It was observed that miRNAs downregulate in cancerous tissues as compared to normal tissues. Several studies have shown the usefulness of circulating miRNAs as diagnostics and prognostics. This reveals the importance of miRNA profiling in cancer diagnosis [42]. Table 1 presents a summary of different types of cancers and miRNA biomarkers reported so far.

While using a global miRNA expression profile in mice liver development, it was observed that miR-500 is an oncofetal miRNA in liver cancer. Its expression was high in fetal liver. Moreover, its expression was downregulated in the developmental process and upregulated during liver cirrhosis. It was reported that miRNA-500 was highly expressed in human cancer cell lines and in around 45% of human HCC tissues. The presence of abundant amounts of miRNA-500 in circulating blood suggests its importance as a novel diagnostic biomarker. The abundance of circulating miRNA-500 in the serum of HCC patients may reflect pathological conditions [47].

Hepatocellular tumors can be classified according to their pathological, clinical, and genetic features by miRNA profiling. MiRNAs are dysregulated in tumors as compared to the non-tumor liver samples. MiRNA-224 was significantly upregulated in both malignant and benign tumors, while miR-422b and miR-122a were downregulated in both types of tumors. There are some miRNAs that behave diffrently in benign and malignant tumors. MiR-200c and miR-203 were downregulated in benign tumors, while miR-224, miR-21, miR-222, miR-10b were upregulated in HCC[34].

Overall, circulating miRNAs are 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 (Table 2). In the current study, for the identification of HBV-HCC or HCV-HCC associated miRNAs, miRNA expression profiling was used [48].

Table 1. Examples of Cancer and Correspondingly 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 a downregulated 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 these levels were significantly reduced after the surgical removal of primary tumors.	[46]
Hepatocellular Carcinoma	Increased MiR-500 level was found in the sera of the HCC patients.	[47]

Table 2. Differential Expression of miRNAs Corresponding to Etiology-related HCC in Published Literature

MiRNA	HCC Etiology	Differential Expression	Reference
Let-7a	HBV associated	Downregulated	[49]
MiR-22	HBV associated	Downregulated	[50]
MiR-29c	HBV associated	Downregulated	[51]
miR-99a	HBV associated	Downregulated	[51]
miR-101	HBV associated	Downregulated	[51]
miR-150	HCV associated	Downregulated	[52]
miR-146	HCV associated	Downregulated	[49]
miR-145	HCV associated	Downregulated	[53]
miR-142	HCV associated	Downregulated	[49]
miR-141	HCV associated	Downregulated	[49]
miR-136	HCV associated	Downregulated	[49]
miR-139	HCV associated	Downregulated	[49]
miR-198, miR-145	HCV associated	Downregulated	[54]
miR-21	HCV and HBV associated	Upregulated	[52]
38. 110. 113			
miR-107	HCV and HBV associated	Upregulated	[56]
miR-135a	HCV and HBV associated	Upregulated	[52]
miR-222	HCV associated	Upregulated	[57]
miR-221	HCV associated	Upregulated	[52]
miR-224	HCV associated	Upregulated	[57]

4. Conclusion

We concluded that the expression of miRNAs is upregulated in the patients of HCC as compared with healthy individuals. In-depth study of miRNAs as genetic biomarkers in the patients of HCC will improve its diagnosis. It will also improve the prognosis of disease at an early stage. This will help to identify suitable and effective therapeutic targets, so as to reduce the chances of the failure of chemotherapy.

Conflict of Interest

The authors declare no conflict of interest.

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