Journal of Bioresource Management

Volume 9 | Issue 3 Article 3

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Ambreen Kanwal
School of Biological Sciences, University of the Punjab, Lahore, Pakistan, ambreen.phd.sbs@pu.edu.pk

Asima Tayyeb
School of Biological Sciences, University of the Punjab, Lahore., asima.sbs@pu.edu.pk

Recommended Citation

Kanwal, A., & Tayyeb, A. (2022). A Review on Serum, Genetic and Mirna Associated Biomarkers for The Early Diagnosis of Hepatocellular Carcinoma (HCC)), *Journal of Bioresource Management, 9* (3).
ISSN: 2309-3854 online
(Received: Dec 23, 2021; Accepted: Jul 13, 2022; Published: Sep 30, 2022)

A Review on Serum, Genetic and Mirna Associated Biomarkers for The Early Diagnosis of Hepatocellular Carcinoma (HCC))

A REVIEW ON SERUM, GENETIC AND MIRNA ASSOCIATED BIOMARKERS FOR THE EARLY DIAGNOSIS OF HEPATOCELLULAR CARCINOMA (HCC)

AMBREEN KANWAL[1] AND ASIMA TAYYEB[1]

[1]*School of Biological Sciences, University of the Punjab, Lahore, Pakistan*

Corresponding author's email: ambreen.phd.sbs@pu.edu.pk

ABSTRACT

Hepatocellular carcinoma is the most devastating complication of liver cirrhosis and diagnosis in earlier stages could be useful in curative interventions. The main aim of this review was to analyze current diagnostic biomarkers which are available for the early diagnosis of hepatocellular carcinoma (HCC). For this purpose, we searched different web databases including Medline/Pubmed. We found multiple significant serum biomarkers for imperative diagnosis including α- Fetoprotein, Des- γ - carboxyprothrombin (DCP), Osteopontin (OPN), Glypican-3 (GPC3), Golgi protein-73 (GP73), Squamous cell carcinoma antigen (SCCA), Annexin A2 (ANXA2) and Heat shock protein 70 (HSP70) but all of these represent low sensitivity and low specificity. Hepatocellular carcinoma diagnosis have also been reported at the genetic level with the help of associated genes such as *p53*, *RAS*, *MERTK* (MER-Tyrosine Kinase), *EGF*, *TGF-β/IGF*, *ALDH2*, CAT, Glutathione S-transferase (*GSTM1*), X-ray repair cross-complementing group1 (*XRCC1*), Receptor tyrosine kinase (*ROR1*), *RASSF1A*, *SOCS1*, *NUF2*, *CXCL2* and Interleukin-1 Gene (*IK-1*). Inhibition of these cancerous genes is under research and could be a novel therapeutic approach in future. Recently, differential diagnosis with miRNA has been found important for hepatocellular carcinoma. Anti-miR-122 could revolutionize the early diagnosis of HCC and has the potential to be marketed for therapy. However, most of the drugs are under clinical trial phase. It is recommended to use them in conjunction with each other so these could be employed as a way to decrease mortality and stigma associated with hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma, diagnosis, genetics, liver cancer, biomarkers.

INTRODUCTION

Cancer refers to a group of diseases involving uncontrolled division of cells and abnormal cell development which can metastases throughout the body. Cancer is a major health problem and cause of mortality worldwide. Hepatocellular carcinoma (HCC) is one of the most devastating types of liver cancer affecting hepatocytes. It is one of the most prevalent malignancies world-wide. While the death rate due to some cancers (such as lung cancer) is decreasing with advanced treatments, death rate due to hepatocellular carcinoma is continuously being increased.

Almost 90 % hepatocellular carcinoma patients also suffer from cirrhosis but only 3 % cirrhotic patients are affected with hepatocellular carcinoma per year (Heimbach et al., 2018). People having cirrhosis are more prone of hepatocellular carcinoma development.

Large scale hepatocellular carcinoma may be viral due to hepatitis B virus (HBV) and hepatitis C virus (HCV) or non-viral due to tobacco smoking, aflatoxin exposure and carcinogens (Baecker et al., 2018). Tobacco smoking, alcohol drugs or other addictive's could disrupt our hepatocytes. A well- known aflatoxin produced by *Aspergillus* play a drastic role in hepatocellular carcinoma development. Carcinogens include nitrites, hydrocarbons and organochlorine pesticides may also play a triggering role for hepatocellular carcinoma development.

Some of the health conditions could also be associated with hepatocellular carcinoma such as obesity. Obese persons have been found with the highest PAF values (around 36.4%). Diabetic patients are more prone to hepatocellular carcinoma as it directly affects liver which is involved in glucose metabolism. The use of oral contraceptives by females also makes them more prone to hepatocellular carcinoma development (Lindberg 1992).

During the past decade, a number of advancements have been done in the field of carcinoma including better medical care, anesthesia, modern surgical techniques and antineoplastic drugs but the overall survival rate of affected individuals suffering with hepatocellular carcinoma have remained dismal. Hepatocellular carcinoma is asymptomatic in the initial stages which makes the diagnosis difficult. It may lead towards intrusiveness, metastasis and reappearance of tumor (Tang et al., 2004).

With the use of biomarkers, tumors can be detected at an early stage in risk population and so curative interventions can be made. Moreover, prognostic markers aid in interrogating the metastatic recurrence of tumor which is useful in treatment and survival of patients. In developing countries with limited resources, designing and usage of accessible biomarkers for hepatocellular carcinoma could revolutionize early diagnosis and treatment of this disastrous illness and can be helpful for people who would otherwise be on stake. The purpose of this review is to provide a brief account of the serum, genetic and miRNA associated biomarkers for the early diagnosis of hepatocellular carcinoma (Figure 1).

α- Fetoprotein (AFP)

α- fetoprotein is glycoprotein belonging to serum albumin family. Gene of this protein is expressed in liver during fetal development and diminishes after birth. AFP gene reactivates in pathological conditions like hepatocellular carcinoma. Plasma concentration 20ng/ml is used as a pathological threshold in humans and 400ng/ml is considered reliable concentration for hepatocellular carcinoma diagnosis (Nakao and Ichikawa 2013). According to guidelines of European and American Association for the Study of Liver Disease, AFP possess low specificity and reduced sensitivity so it could not be used as diagnostic marker. AFP-L3 % is the most commonly used glycosylated form of AFP in early diagnosis of tumor when its size is 2cm as its sensitivity depends on tumor size. AFP shows false negative results in 40 % of hepatocellular carcinoma patients in early stage and 15-30 % patients possess normal AFP levels even in advanced stages of cancer which proved it less efficient biomarker (Cheng et al., 2014).

Des- γ -Carboxyprothrombin (DCP)

DCP is inactive abnormal from of prothrombin and also known as PIVKA-II (protein induced by vitamin K absence or antagonist-II). It forms as a result of impaired carboxylation of 10 glutamic acid residues. In 91% hepatocellular carcinoma patients, DCP is present in their serum suggesting its role as a prognostic marker (Liebman et al.,

1984). Different studies have described sensitivity and specificity of elevated DCP concentrations in different stages of hepatocellular carcinoma patients. DCP concentration vary according to the disease progression and tumor size (Mohammad et al., 2008). DCP is known to be a favorable biomarker in cases of AFP negative hepatocellular carcinoma.

Osteopontin (OPN)

OPN is a calcium binding extracellular glycoprotein. This cytokine has important role in bone mineralization and various abnormal physiological activities like chronic inflammation therefore it is expressed in malignancies like hepatocellular carcinoma.

HCC Diagnosis at Protein, Genetic and miRNA level

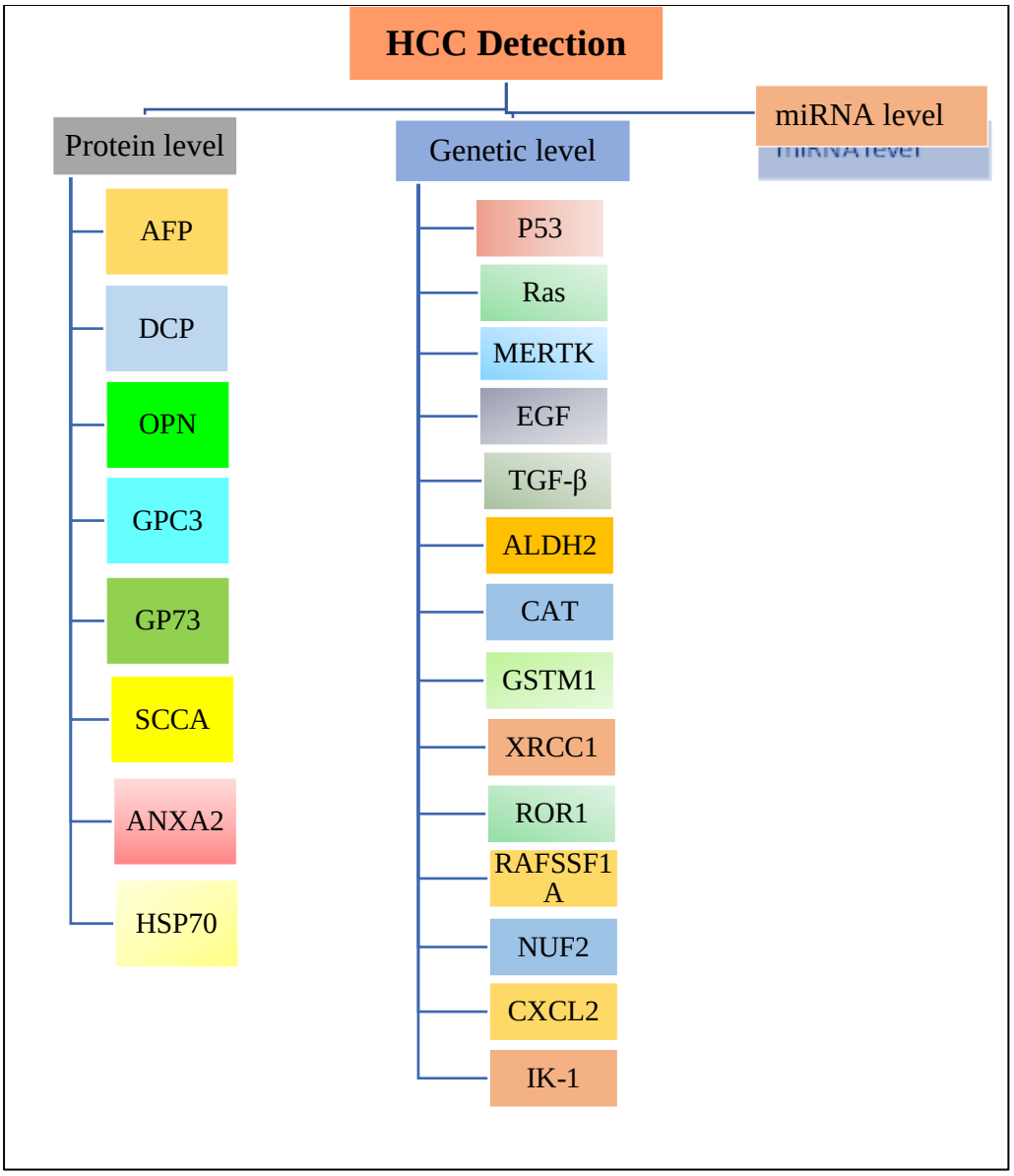

Figure 1: Biomarkers for Hepatocellular carcinoma (Caldwell et al., 2004).

OPN has been designated as prognostic marker for progression of hepatocellular carcinoma (Matsui et al., 2004). OPN overexpression has been found linked with metastatic potential of HCC. OPN has also been found important in HCV related hepatocellular carcinoma. AFP shows better diagnostic capability as compared to OPN but osteopontin possess higher sensitivity in identification of hepatocellular carcinoma at early stages. A combination of AFP and OPN could be more useful and reliable (Al-Zoubi et al., 2017).

Glypican-3 (GPC3)

GPC3 is a cell membrane anchoring protein and belongs to heparin sulfate proteoglycans (HSPGs) family. It shows prominent role in cell growth, differentiation and cancerous cell proliferation by Wnt and Hedgehog pathways (Scaggiante et al., 2014). GPC3 has increased expression in hepatocellular carcinoma cells as compared to normal, unaffected tissues or in the sera of individuals suffering with hepatocellular carcinoma. Higher expression of GPC3 protein is associated with high tumor and vascular invasion. It is similar to AFP but reveals low sensitivity and increased specificity (Xu et al., 2013). Another study reported the increased sensitivity and higher specificity of GPC3 in the sera of hepatocellular carcinoma patients as compared to AFP, further suggesting the promising results while using a combination of both biomarkers (El-Saadany et al., 2018).

Golgi Protein-73 (GP73)

GP73 also referred as Golgi phosphoprotein 2, is transmembrane glycoprotein consisting of 73 kDa. It is found overexpressed in fibrosis, carcinogenesis and hepatocellular carcinoma patients as compared to normal controls. GP73 is reported as diagnostic marker of hepatocellular carcinoma due to its elevated expression in affected individuals. After surgical hepatectomy, the diagnostic capability of GP73 is far greater than AFP and is a beneficial biomarker for the follow-up of hepatocellular carcinoma patients. Limitations include serum GP73 increase only in hepatocellular carcinoma patients having cirrhosis, but not in patients without cirrhosis making it an unsuitable hepatocellular carcinoma diagnosis biomarker (Liu et al., 2017). Serum GP73 can be used as a potent biomarker in predicting liver inflammation in chronic HBV patients. A recent study has reported the use of both serum and mRNA of GP73 as diagnostic marker for early detection of hepatocellular carcinoma (Farag et al., 2019). To solve the controversy of GP73 as biomarker, a meta-analysis has shown its predictive role for hepatocellular carcinoma invasion and metastasis (Zhang et al., 2019).

Squamous Cell Carcinoma Antigen (SCCA)

SCCA is a high molecular weight serine protease inhibitor which play important role in epithelial cancers. Its two isoforms SCCA1 and SCCA2 are at higher level in hepatocellular carcinoma patients as compared to normal ones. The third genetic isotype of SCCA characterized by G351A polymorphism has been found in some cases of hepatocellular carcinoma. Due to its presence in hepatocellular carcinoma, it is documented as possible diagnostic biomarker (Soyemi et al., 2012). Important role of SCCA in diagnosis of hepatocellular carcinoma has been found with sensitivity of 77.6% and specificity of 84.4%. This biomarker does not have the fair diagnostic value for hepatocellular carcinoma making it not the perfect one instead of

other biomarkers like AFP, GP73 etc. SCCA possess higher sensitivity while AFP has higher specificity, so a combination of the two biomarkers make a good predicting test with better sensitivity for hepatocellular carcinoma (Abdelhamid et al., 2020).

Annexin A2 (ANXA2)

ANXA2 is a 36 kDa cytoskeletal protein which binds with calcium, present at the surface of the endothelial cells and on different kinds of cancerous cells. ANXA2 plays significant role in regulatory activities like cell invasion, migration, adhesion and proliferation. It has been reported in different types of cancers as in breast, liver, ovary and hepatocellular cancer (Lokman et al., 2011, Mohammad et al., 2008). As compared to AFP, ANXA2 has been utilized as a serological biomarker for the early diagnosis of hepatocellular carcinoma with advanced sensitivity and acute specificity. Serum ANXA2 has been used as diagnostic marker with sensitivity of 74% and specificity of 88% (Shaker et al., 2017). Elevated expression of ANXA2 in both AFP positive and AFP negative patients complements that this combination could form a better diagnostic marker (El-Gezawy et al., 2018).

Heat Shock Protein 70 (HSP70)

HSPs are cellular molecules belonging to protein family that are expressed under the effect of stressors such as hypoxia, nutrient deficiency, starvation and carcinogenesis. Among HSPs, the most common is a molecular chaperon Heat shock protein 70 (HSP70) whose level is increased as a result of cell survival protection, heat and other stressors. The level of HSP70 is regulated by heat shock factor-1 (HSF-1). HSF-1 can block apoptosis at different levels and its role in cancer is might be due to its anti-apoptotic activity. HSP70 and HSF-1 are found to be involved in hepatocellular carcinoma metastasis. Affected individuals suffering from hepatocellular carcinoma and liver cirrhosis, serum levels of HSP70 are generally greater as compared to normal individuals (Gehrmann et al., 2014). HSP70 expression is upregulated during early stages of hepatocellular carcinoma and so it is used as potential sensitive biomarker. HSP70 in combination with other markers like glutamine synthetase could form putative diagnostic biomarker for hepatocellular carcinoma (Jiang et al., 2020). GPC3 and HSP70 appear to be useful as individual markers for hepatocellular carcinoma arising in non-cirrhotic livers (Uthamalingam et al., 2018).

HCC Diagnosis at Genetic Level

Mutation in driver genes and pathways convert normal cells into tumorous and allow them to proliferate without constraints (Zhang et al., 2018). Hepatotoxic stress, viral infections or carcinogens can proliferate mutations in tumor-suppressor genes (Tannapfel and Wittekind 2002). Changes in the promoter region, gene amplification or point mutations may result disrupting cell cycle leading towards development of metastatic cancer. Thus, inhibition of cancerous genes from outside sources could be a novel therapeutic approach for the treatment of various types of cancer (Kanda et al., 2015).

p53

p53 mutations have been found in 30 % of hepatocellular carcinoma cases worldwide out of which most mutations are somatic. A hotspot mutation for hepatocellular carcinoma is p.Arg249Ser (guanine-to-thymine transversion) (Qin et al., 2020). Occurrence of this "hotspot" mutation is extremely low in hepatocellular carcinoma patients who are from Australia, Japan, United States, or Europe. Among 664 hepatocellular carcinoma patients, only three mutations (two in Japan and one in Europe) have been recognized which revealed that other genes might be intricate in the process of hepatocellular carcinoma development (Tannapfel and Wittekind 2002)**.**

RAS Gene

Three human Ras genes (*K-RAS, N-RAS* and *H-RAS*) encodes small K-Ras4A, K-Ras4, N-Ras and H-Ras guanosine triphosphate (GTP) binding proteins which regulate the growth of cells, apoptosis and cellular differentiation. Single amino acid substitutions at K-ras codon 61, H-ras codon 13 or N-ras codon 12 consequences in the development of mutant Ras proteins which are insensitive to GAP (Clark et al., 1993). As a result, mutant oncogenic Ras proteins remain active and bound to GTP. Overexpression of Ras has been demonstrated in hepatocellular carcinoma. Likewise, inhibitors of Ras pathway are downregulated in HCC. K-Ras codon 12, H-Ras codon 12 and N-Ras codon 6 are the most prevalent mutations of Ras proteins. So these mutations in *RAS* genes can be used as biomarker tool in cancer diagnosis (Chen et al., 2020)**.**

MERTK (MER-Tyrosine Kinase)

MERTK resides on chromosome 2 and plays important role in controlling tumor associated macrophages (TMA). *MERTK, rs6726639* causes progression of hepatocellular carcinoma in patients. These patients have achieved sustained virologic response (SVR) which can be treated with antiviral substances. The AA allele of *rs6726639 MERTK* is considered to be responsible for higher risk of developing hepatocellular carcinoma in patients with cirrhosis after HCV eradication by DAAs. This discovery can be useful in hepatocellular carcinoma early diagnosis by considering this mutation an important biomarker of hepatocellular carcinoma (Di Marco et al., 2019).

EGF

SNP *rs4444903* of *EGF*, also known as +61, regulates the production of EFG. This polymorphism is present in the promoter region of epidermal growth factor *EGF*. The *rs4444903* (G) allele has been found to be responsible for increasing EGF levels than the (A) allele. Significant association of this polymorphism with hepatocellular carcinoma development has been found so it can be used as a biomarker if more research is carried out on this perspective (Suenaga et al., 2013).

TGF-β/IGF

Transforming growth factor (TGF) β plays a significant role in apoptosis, growth rate and cell cycle. In 10% of hepatocellular carcinoma cases, mutations in

SMAD2 and *SMAD4* cause genetic changes of TGF-β pathway. Mutation in TGF-β receptor (*TGF-β1RII*) gene causes hepatocellular carcinoma development by inhibiting TGF-β signaling. Insulin-like growth factor 2 receptor/ Mannose-6-phosphate (IGF2R/M6P) activates *TGF-β*. In about 30% hepatocellular carcinoma patients, *M6P/IGF2R* mutation plays significant role in hepatocellular carcinoma development. Different genes of TGF-β/IGF pathway may be mutated in hepatocellular carcinoma which hampers its use for diagnosis (Tannapfel and Wittekind 2002).

ALDH2

Mitochondrial aldehyde dehydrogenase enzyme is encoded by *ALDH2* gene. Significant association has been found between ALDH2 and hepatocellular carcinoma development but there is ultimate need to further investigate these associations (Kato et al., 2003).

CAT

CAT gene encodes antioxidant enzyme catalase. Polymorphism of this gene SNP *rs1001179*.C>T has been found to be associated with hepatocellular carcinoma development but it needs further investigation (Ezzikouri et al., 2010, Nahon et al., 2012).

Glutathione S-transferase (GSTM1)

GSTM1 encodes Glutathione S-transferase enzyme belonging to Mu class of enzymes. These enzymes play a significant role in detoxification of electrophiles and in carcinogenesis. *GSTM1* is present in a gene cluster on chromosome 1p13.3 and is found to be highly polymorphic. A deletion of this gene has been found to be associated with hepatocellular carcinoma (Asim et al., 2010, El-Moneim et al., 2008).

X-ray Repair Cross-Complementing Group1 (XRCC1)

XRCC1 plays essential role in hepatocellular carcinoma development. The most devastating mutations causative for hepatocellular carcinoma development have been found among chronic hepatitis C patients c.1254C>T (*rs2293035*) and c.1517G>C (*rs139599857*) in *XRCC1* (Arafa et al., 2019). Further research is needed for using this polymorphism as biomarker for hepatocellular carcinoma diagnosis.

Receptor Tyrosine Kinase (ROR1)

Receptor tyrosine kinase (ROR1) has functional contribution in hepatocarcinogenesis. In human and mouse hepatocellular carcinoma cell lines, ROR1 antibodies are utilized for the localization of ROR1 protein. Knockdown of *ROR1* declines proliferation and enhances apoptosis. Hence it shows *ROR1* can have diagnostic value in hepatocellular carcinoma and can be proved as an important biomarker (Cetin et al., 2019).

RASSF1A and SOCS1

DNA methylation is considered as most significant genetic modification in human cancers. Instead of performing biopsy, methylation identification can be a

significant prognostic biomarker for hepatocellular carcinoma (HCC). In 38% and 40% of affected individuals suffering with hepatocellular carcinoma, methylation patterns of *SOCS-1* and *RASSF1A* respectively, have been found causative. So *SOCS-1* and *RASSF1A* methylation status may be used a biomarker tool for hepatocellular carcinoma (Pasha et al., 2019)**.**

NUF2

Early tumor recurrence after getting treated is major challenge in hepatocellular carcinoma so *NUF2* that encodes kinetochore protein is considered an important biomarker for diagnosis of early recurrence of hepatocellular carcinoma. In a study in which whole genome expression microarrays have been performed on 64 primary hepatocellular carcinoma tumors, *NUF2* has been found considerably related with early recurrent hepatocellular carcinoma tumors (Wang et al., 2019)**.**

CXCL2

Epigenetics causes shushing of tumor suppressor genes by methylation of promoter CpG islands. After treatment with DNA demethylating agent, 5-aza-2′-deoxycytidine, the *CXCL2* expression and methylation has been found significantly down regulated in hepatocellular carcinoma tissues. Hence *CXCL2*, may be used as an important biomarker for diagnosis and treatment of hepatocellular carcinoma (Subat et al., 2019).

Interleukin-1 Gene (IK-1)

Interleukin family is a group of cytokines that regulate immune and inflammatory responses. In 274 Japanese HCV patients, *IK-1* polymorphism has been studied along with its association with occurrence of hepatocellular carcinoma by applying standard PCR-based genotyping techniques. IL-1-511/-31 haplotype C-T and IL-1-31 genotype T/T has been found significantly associated with hepatocellular carcinoma occurrence (Wang et al., 2003).

Hepatocellular Carcinoma Diagnosis at Micro-RNA level

Human genome encodes more than one thousand types of micro-RNA (miRNA) involved in the process of gene silencing. miRNA pair up with its target messenger RNA (mRNA) molecules due to complementary sequence within the target mRNA. This pairing induces degradation of mRNA either by its cleavage or by chopping off its poly-A tail and results in decreased expression of a target gene or mRNA into protein (Xu et al., 2013).

miRNAs are being recognized as potential biomarkers in diagnostic studies as they are quite stable and can be detected in clinical samples such as bodily fluids, urine, serum, blood and cerebrospinal fluid. Different disease conditions, particularly cancer, leads to deviation from normal expression of various miRNAs making them a significant biomarker in diagnosis of hepatocellular carcinoma. miRNA are also referred as oncomirs due to their association with many cancers as abnormal expression of miRNA may serve in early diagnosis of cancers (Su et al., 2019).

Expression of characteristic miRNAs has been found as a promising tool in prediction, diagnosis, and prognosis of therapeutic responses in hepatocellular carcinoma. As the hepatocellular carcinoma progresses, unique pattern of miRNAs

expression vary gradually and many tumor suppressing genes are recognized as their potential target sites. Differential miRNA expression in hepatocellular carcinoma is studied extensively and demonstrated comprehensively, enabling them to diagnose hepatocellular carcinoma at early stages from samples including blood, body fluids and tissue specimens. Comparison of various miRNAs in normal healthy liver tissues and tissue specimen from cancerous liver has demonstrated miR-122 as the most abundant miRNA found in healthy liver tissues whose level is downregulated during the progression of hepatocellular carcinoma (Liang et al., 2020).

Another miRNA that express in normal liver tissue is miR-199a/b whose level has been found downregulated during hepatocellular carcinoma development leading to poor survival rate (Lou et al., 2020).

Variation in expression of miRNAs in serum can also be detected. Level of miR-122 in the serum of patients suffering from hepatocellular carcinoma has been found upregulated. Chemotherapy in these patients causes drop in serum expression of miR-122 and hence serve as potent biomarker for hepatocellular carcinoma severity during its treatment (Lou et al., 2020). A combination of three miRNAs: let-7f, miR-375 and miR-25 discriminate serum sample of hepatocellular carcinoma from healthy controls. Deviation in serum level of seven miRNAs , miR-505, miR-192, miR-145, miR-143, miR-29a, miR-29c, and miR-133a can detect hepatocellular carcinoma with much better sensitivity as compared to other biomarkers (Fu et al., 2019).

Decreased expression of miR-150 in the serum of HBV-related hepatocellular carcinoma patients has indicated considerable low survival rate. Hepatocellular carcinoma and chronic hepatitis can also be differentiated through the change in profile of circulating miR-199a, miR-16 and miR-195 (Sui et al., 2019). However, a reduced serum level of some miRNAs such as miRNA-21, miRNA-122 and miRNA-223 is involved in Hepatitis as well as hepatocellular carcinoma.

It is very obvious from above mentioned findings to establish the role of circulating miRNAs as potential diagnostic marker in hepatocellular carcinoma. Figure 2 demonstrates distinctive upregulation or downregulation of various circulating miRNAs in hepatocellular carcinoma patients.

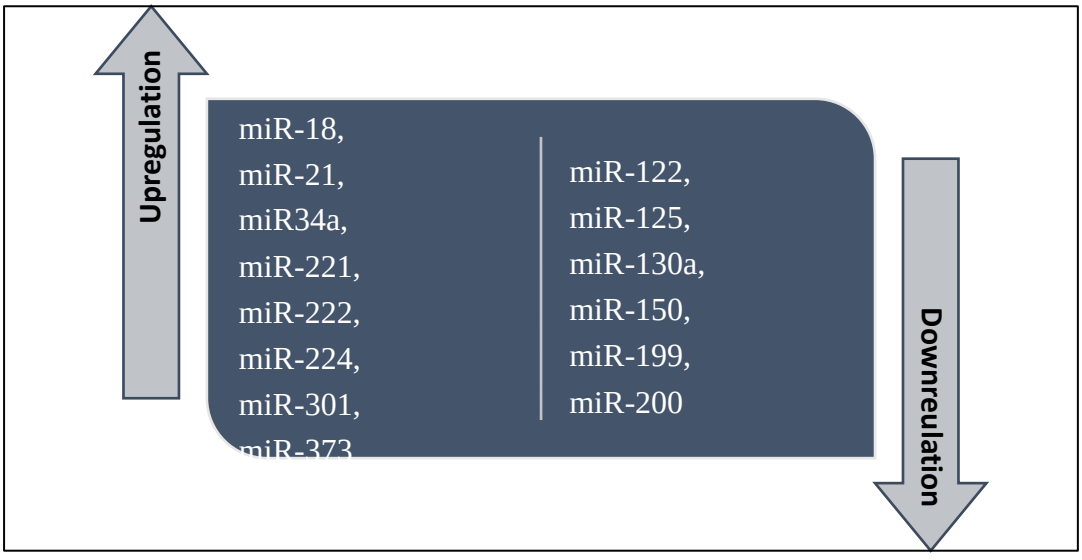

Figure 2: Summary of some circulating miRNAs.

miRNA that are upregulated are shown on left side and miRNAs that are downregulated are indicated on right side.

Apart from diagnosis, miRNAs have tremendous potential to be used as prognostic markers. They may help in determination of tumor size, metastasis stage of hepatocellular carcinoma, recurrence and even the survival rate of patient. Downregulation of miR-100 and miR-22 along with the upregulation of miR-221 depicts the distant metastasis in primary hepatocellular carcinoma tissues (Fu et al., 2019). Advancement of tumor can be correlated with the change in expression of miR-222 in the serum (Eguchi et al., 2018). A higher expression of miR-182, miR-155, miR-372 and miR-25 in hepatocellular carcinoma tissues portrays lesser survival rate. However, the decreased expression level of miR-29a-5p, miR-100, miR-29, miR-101 and miR-148 is indicative hepatocellular carcinoma treatment leading to increased survival chances (Cetin et al., 2019).

CONCLUSION

This study concludes that different biomarkers could be employed due to their differential expression. Serum, genetic or miRNA associated biomarkers have the remarkable potential for the early diagnosis of hepatocellular carcinoma (HCC) but these could be used in conjunction with each other.

AUTHOR'S CONTRIBUTION

AK wrote the original manuscript and AT supervised the work.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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