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Diagnostic Potential of miRNA in MRI Diagnosed Patients with Hepatocellular Carcinoma

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Abstract:

Background: *Hepatocellular carcinoma (HCC) is one of the most common and lethal cancers worldwide. Early detection of HCC is necessary for more effective planning of management strategies. Detecting HCC at such an early clinical stage is difficult using traditional diagnostic methods as AFP and ultrasonography. Recent advances in MRI techniques as DWI and PWI are now promising to be more useful than the other techniques for such assessments. MicroRNA (miRNA), is a class of short noncoding RNA molecules, controlling approximate one third of the protein-coding genes by posttranscriptional regulation of gene expression and can directly or indirectly affect almost all cellular pathways. Specific miRNA aberrations involved in cancer development and progression have been identified. Therefore, many miRNAs are proposed as promising biomarkers for early detection of HCC. The aim of the present study was to investigate the diagnostic potential of miRNA in early cases of HCC diagnosed by advanced MRI techniques. Subjects and methods: 28 HCC patients diagnosed using advanced MRI techniques and 10 healthy controls were enrolled in this study. microRNA was extracted from patients and control group sera and detection of miR-122 was done by real time PCR. In addition AFP was measured in all subjects. Our data indicated that expression levels of miR-122 in serum were significantly higher in HCC patients than healthy controls ($p<0.001$). In addition, miR-122 was elevated in all patients group in contrast to*

AFP which was normal in 18% of patients. Which indicates that miR-122 have higher sensitivity than AFP in diagnosing HCC.

Key words: miRNA, MRI diagnosed patients, hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and lethal cancers worldwide. The strongest risk factors for HCC are chronic hepatitis B and C viral (HBV, HCV) infection, as well as alcoholic liver disease. One of the main reasons for the lethality of HCC is the lack of diagnostic markers for early detection of the disease [(1)]. Early detection of HCC is necessary for more effective planning of management strategies like tumor resection, liver transplantation, tumor ablation and radiofrequency [(2)] .Detecting HCC at such an early clinical stage is difficult using traditional diagnostic methods; thus, molecular diagnostics that make use of biomarkers represent a large unmet need in HCC care[(3)]. Screening tests for HCC are classified to serological and radiological tests. The serological tests includes alpha feto protein (AFP) and prothrombin induced by vitamin K absence II (PIVKA II).While using AFP 20 ng/ml the optimal balance between the sensitivity and specificity yet at this level the sensitivity is only 60%.The most widely used radiological test for surveillance is ultrasound with sensitivity 65% and specificity about 90%[(4)] . Recent advances in magnetic resonance imaging (MRI) techniques as DWI and PWI are now promising to be more useful than computed tomography (CT) and ultrasonography (US) for such assessments because it provides better soft tissue contrast and a more accurate depiction of different tissue properties, thus it detects HCC in an earlier stage of the disease[(5)].

MicroRNA (miRNA), is a class of short noncoding RNA molecules, controlling approximate one third of the protein-

coding genes by posttranscriptional regulation of gene expression and can directly or indirectly affect almost all cellular pathways[6] . The transcription of miRNA is guided by RNA polymerase (pol II) regulators, which are often deregulated in case of liver pathologies. In HBV- and HCV-associated pathogenesis, c-Myc along with viral oncoproteins modulates miRNA expression, to create an oncogenic milieu facilitating the binding of transcriptional repressor complexes to miRNA promoters to allow its sustained expression[7].Specific miRNA aberrations involved in cancer development and progression have been identified. Therefore, many miRNAs are proposed as promising biomarkers for early detection of HCC and accurate predictions of prognosis, as well as targets for treatment[8].

The aim of the present study was to investigate the diagnostic potential of miRNA in early cases of HCC diagnosed by advanced MRI techniques.

PATIENTS AND METHODS

Patients:

Seventy one patients were included in this study recruited from the patients attending outpatient clinic of Ain Shams university hospital HCC unit. They all were patients with HCV related chronic liver disease with hepatic focal lesion or suspected malignancy. The study was conducted in accordance with the World Medical Association Declaration of Helsinki for human subjects and the study was approved by the ethics committee of the Faculty of Medicine Ain Shams University, and all participants gave us their written informed consent before enrolment.

Methods:

All the 71 patients were subjected to the routine follow up screening tests done to HCV patients, in addition to CT to improve the sensitivity of the screening.

Laboratory investigations: including liver enzymes AST and ALT, bilirubin and AFP.

Radiological investigations: including ultrasonography of the abdomen and triphasic computed tomography (CT).

According to the results the patients were divided into two groups:

Group I: included the patients with laboratory and radiological evidence of HCC (n=26).

Group II: included the patients with equivocal hepatic focal lesion (n=45).

MRI examination: including triphasic contrast enhanced MRI, DWI and PWI were done to the second group to identify and characterize the focal lesion (as it provides better soft tissue contrast and a more accurate depiction of different tissue properties than CT).

MRI examination was done at Ain shams University radiology department using a 1.5 tesla Magnetic Resonance (Philips, Achieva): an 8 channel phased array coil was used for acquisition of liver images.

The study protocol basically included: Axial T1WIs, axial and coronal T2WIs, gradient echo sequences including inphase and outphase sequences, coronal balanced fast field echo sequence (BEFE), axial heavy T2WIs and dynamic multiphase thrive T1WIs acquired before and after a gadolinium contrast agent (Gd-DTPA 0.1 ml/kg – Bayer Schering, Germany).

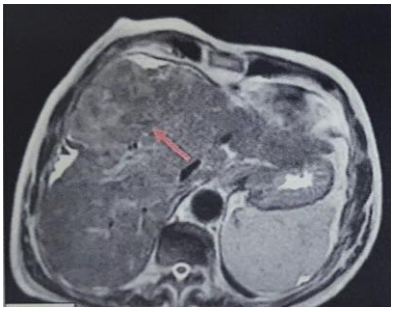

Figure (1): A large exophytic HCC displaying hyper intense signal in T2wi.

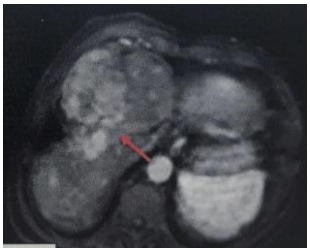

Figure (2): Gadolinium enhanced image showing arterial enhancement of the tumor in the late arterial phase.

Group II of patients was further divided according to MRI examination into:

a) HCC patients group: included 28 patients diagnosed by MRI examination to be affected by HCC.
b) Non HCC group: included 17 patients ; 5 of them were dysplastic nodules, 6 were regenerating nodules, 3 were haemangiomas, 1 was fatty spraying and 1 was AV shunt.

The standard reference for diagnosis was either by biopsy or excision and histopathological verification or follow up of the lesion after 3 and/or 6 months.

Micro RNA detection:

Detection of miRNA-122 was done for group II(b)(the group of patients diagnosed by MRI to have HCC. They were 28 patients (19 males, 9 females). Ten age and sex matched apparently healthy subjects were included as a control group.

Serum preparation and RNA extraction

Ten milliliters of peripheral blood were collected from patients and controls. Cellular components were removed by centrifugation and the sera were stored at -80 °C until use. RNA was isolated using miRNeasy Mini Kit (Qiagen, Germany). 700 µl of the serum was pipetted into an RNeasy Mini spin column in a 2 ml collection tube and centrifuged at 10,000 rpm for 15 sec at room temperature (15–25°C) ,the flow through was discarded and this step was repeated using the remainder of the sample and the flow through was discarded. 500 µl Buffer RPE was pipetted onto the RNeasy Mini spin column, and centrifuged for 15 sec at 10,000 rpm. Another 500 µl Buffer RPE was added to the RNeasy Mini spin column, and centrifuged for 2 min at 10,000 rpm. The RNeasy Mini spin column was transferred to a new 1.5 ml collection tube and 50 µl RNase-free water was pipetted directly onto the RNeasy Mini spin column membrane and centrifuged for 1 min at 10,000 rpm.

Real-time quantitative RT-PCR analysis of miRNAs

A cancer-associated miRNA miR-122 was chosen on the basis of its reported relevance to HCC Koberle et al. (2013)[9] and Xu et al. (2012)[10].Small RNA U6 was used as a reference gene for miRNA expression analysis (reference gene is expressed in all cells under normal and patho-physiological conditions). miRNA expression was quantified in real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) systems using TaqMan microRNA assays according to the manufacturer's protocols (Applied Biosystems), which included two steps: RT reaction and TaqMan real-time PCR assay. RT reactions were performed as follows: the reagents were allowed to thaw on ice, in a polypropylene tube the master mix was prepared as follows: for each reaction we pipetted 0.15µL dNTP mix (100 mM total), 1µL RT enzyme (50U/µL), 1.5 µL 10× RT buffer, 0.19 µL RNase inhibitor(20U/ µL) and 4.16 µL Nuclease

free water, then 7 μL of the master mix was transferred into a reaction tube, we then added 5 μL RNA and 3 μL of the RT 5× TaqMan microRNA primers (Applied Biosystems) , mix gently without vortex. For each sample two reaction tubes were used, in the first one we added specific miR-122 primers and in the second tube we added specific small RNA U6 primers (the first tube is used to amplify miR-122 and the second tube is used to amplify small RNA U6), as microRNA detection is done in the form of relative expression of the target to the reference gene. The reaction mixture was incubated for 30 min at 16°C and 30 min at 42°C, followed by 5 min incubation at 85°C to inactivate the RTase enzyme. RT products were subjected to miRNA expression assay for real-time quantitative PCR, PCR reaction mixture included 2 μL of RT product, 1 μL of 20× TaqMan microRNA primers (Applied Biosystems) and 10 μL of 2×TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR cycling parameters were 95°C for 15 sec followed by 60°C for 30 sec for 40 cycles. In each PCR run we enter a control sample, the same steps are done for the control (2 tubes one for detection of miR-122 and one for detection of small RNA U6 Real-time quantitative PCR was performed in a Stratagene Real-Time System .The cycle threshold (CT value) was defined as the number of cycles required for the fluorescent signal to cross the threshold in quantitative PCR. The expression of miR-122 is defined as RQ, the RQ is calculated as follows:

$$\Delta CT(sample)=Target\ CT(sample)-reference\ gene\ CT(sample)$$
$$\Delta CT(control)=Target\ CT(control)-reference\ gene\ CT(control)$$
$$\Delta\Delta CT= \Delta CT(sample)- \Delta CT(control)$$
$$RQ=2^{-\Delta\Delta CT}$$

The results (RQ) of the HCC group were compared to the results of the control group to identify weather miR-122 is over expressed, under expressed, or there is no difference in expression between the two groups. In order to define if there is

significant differential expression in miR-122 level between the two groups, so it can be used in the diagnosis of HCC.

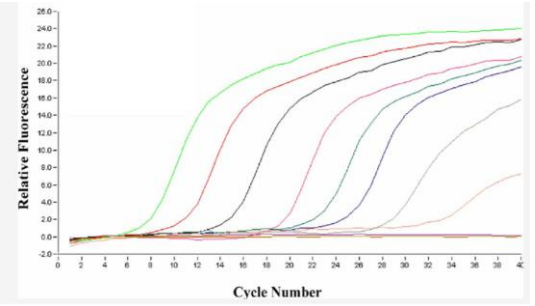

Figure (3): qPCR amplification curve showing the threshold cycle (CT) of different samples.The green curve (the first one from the left side) has the lowest CT as it was the first sample to cross the threshold line which indicates that this sample has the highest concentration of the target (miR-122),and the pink curve (the first one from the right side) has the highest CT as it was the last sample to cross the threshold line which indicates that this sample has the lowest concentration of the target.

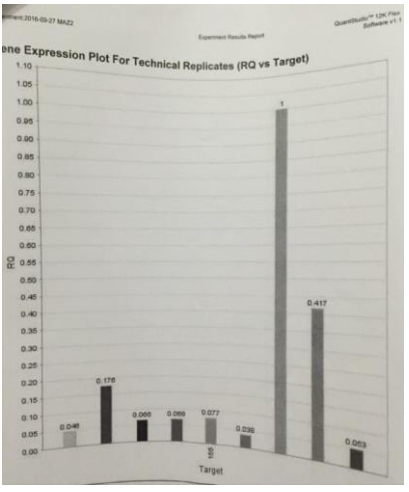

Figure (4): Gene expression plot showing the RQ of miR-122 in different samples.

Statistical Analysis:

Data were analyzed using Statistical Program for Social Science (SPSS) version 20.0. Quantitative data were expressed as mean ± standard deviation (SD). Qualitative data were expressed as frequency and percentage.

The following tests were done:

- Independent-samples t-test of significance was used when comparing between two means.
- Probability (P-value):

1. P-value $\leq$0.05 was considered significant.
2. P-value $\leq$0.001 was considered as highly significant.
3. P-value >0.05 was considered insignificant.

- Calculation of sensitivity, specificity, positive and negative predictive values.

RESULTS

Patients characteristics

A total of 38 participants including 28 HCC patients (diagnosed by MRI) and 10 normal control subjects were recruited in this study (Table 1).The age median in the control and HCC group was 49 and 53 years respectively. The sex distribution was 7 males and 3 females in the control group, 19 male and 9 females in the HCC group. There were no significant differences of age and sex between patients and controls.

On the other hand, the patients and the control group had statistically significant different laboratory results for AST, ALT and bilirubin with the HCC group being higher (P<0.05) (Table 2).

HCC patients produced a wide range of AFP values from normal to 1,197 ng/ml, mean ±SD (63±239).Normal AFP level up to 10 ng/ml is present as many as 18%(n=5) of patients, AFP level between 10 and 20 ng/ml in 7%(n=2) of patients and AFP>20 ng/ml in 75%(n=21) of patients with 10.7%(n=3) of patients having AFP>400 ng/ml. While in the control group ,

AFP mean±SD was (4±1.3), with highly statistically significant difference between the two groups (P<0.001) (Table 2&Figure 4).

Table (1): Age and sex distribution of the participants

	Control n=10	**HCC n=28**
Age, median, y	49	53
Men, n (%)	7 (70%)	19 (67%)

Table (2): Laboratory findings of patients and controls.

	Control n=10	**HCC n=28**	**t-test**	**P value**
AST (U/L)	17±3	47±5	2.91	<0.05
ALT (U/L)	21±4	53±4	2.71	<0.05
Bilirubin (mg/dl)	0.4±0.15	1.8±0.4	2.82	<0.05
AFP	4±1.3	63±239	7.60	<0.001

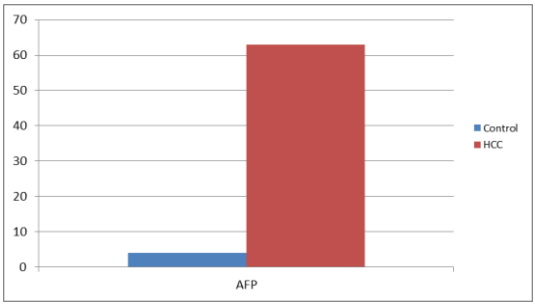

Figure (4): Bar graph comparing the mean of AFP values in the control and the HCC groups.

Identification of HCC associated miRNA in serum:

The goal of the present study was to explore the potential use of serum miRNA as biomarkers for HCC. RT-qPCR assay was done to quantify miR-122 in serum among patients and controls. The patients in the present study were diagnosed as HCC patients using advanced MRI techniques after failure of the conventional methods (US &CT) to reach a definitive diagnosis.

Mean expression level of miR-122 (RQ mean) in HCC group was (1.327±0.415) and in control group was (0.149±0.076)

with highly statistical significant difference between the two groups (P<0.001) (Table 3).

Table (3): Comparison of miR-122 expression levels in control group versus HCC patients.

	Control	HCC	t-test	P-value
miR-122(RQ)	0.149±0.076	1.327±0.415	4.612	<0.001

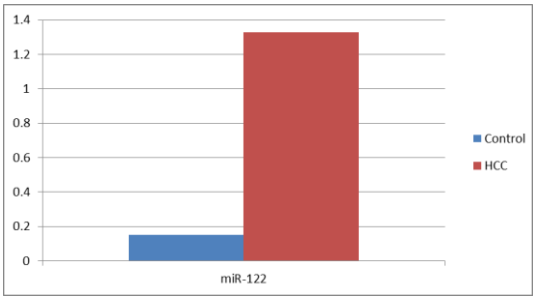

Figure (5): Bar graph comparing the mean of miR-122 RQ values in the control and the HCC groups.

The sensitivity and specificity of AFP and miR-122 as regards diagnosing HCC were calculated and the results denoted that AFP sensitivity (true positive rate) was 82% and specificity (true negative rate) was 100%, while miR-122 sensitivity was 100% and specificity was 100%.The positive predictive value (probability that the disease is present when the test is positive) of both parameters was 100%,while the negative predictive value (probability that the disease is not present when the test is negative) of AFP was 66.67% and of miR-122 was 100%.Table (4).

Table (4): comparing the sensitivity, specificity, positive predictive value and negative predictive value of AFP and miR-122 in diagnosing HCC.

	AFP	miR-122
Sensitivity	82%	100%
Specificity	100%	100%
Positive predictive value	100%	100%
Negative predictive value	66.67%	100%

DISCUSSION

MicroRNAs (miRNAs) are evolutionary conserved small noncoding RNAs that regulate gene expression by mediating posttranscriptional silencing of target genes. Since miRNAs are involved in fine-tuning of physiological responses, they have become of interest for diagnosis and therapy of a number of diseases. Moreover, the role of dysregulated miRNAs in maintaining the malignant phenotype has profound implications for cancer therapy [(11)].

Hepatocellular cancer (HCC) is a primary malignancy arising within the liver. This disease is the third most common cause of cancer deaths in men worldwide. The incidence and mortality of HCC are increasing in many countries [(12)]. There is now mounting evidence indicating that specific miRNAs may act as oncogenes or tumor suppressors by directly or indirectly controlling the expression of key proteins involved in hepatocarcinogenesis[(13)]. Hence, further validation of potential important miRNAs involved in initiation, progression, and metastasis provides valuable insight for the diagnosis and therapy of patients with HCC[(14)].

Serum α-fetoprotein (AFP) has been used as a diagnostic biomarker of hepatocellular carcinoma, however the sensitivity and specificity of detection are poor. Approximately 20% of HCC patients with low AFP level are poorly diagnosed [(15)].

In the present study, measuring the level of AFP revealed that HCC patients produced a wide range of AFP values from normal levels up to 1,197 ng/ml, mean ±SD (63±239). Normal AFP level up to 10 ng/ml is present as many as 18%(n=5) of patients, AFP level between 10 and 20 ng/ml in 7%(n=2) of patients and AFP>20 ng/ml in 75%(n=21) of patients with 10.7%(n=3) of patients having AFP>400 ng/ml. While in the control group , AFP mean±SD was (4±1.3), with highly statistically significant difference between the two groups ($P<0.001$). This is in accordance with Motawi et al. (2015)[(16)]

who found that nearly one third of early HCC patients are missed by AFP analysis and that it is also elevated in patients with chronic hepatitis and cirrhosis, which decreases its sensitivity and specificity in diagnosing HCC.

In the present study, we measured the level of miR-122 in the serum of patients undergoing RF procedure for treatment of hepatocellular carcinoma. We found that the level of miR-122 was statistically significantly higher in patients than in control group ($P < 0.001$).

This is in accordance with the results stated by Hung et al.(2016)[13] who found that miR-122 and let-7b, were upregulated in the serum of HBV-related early HCC patients, also Xu et al. (2011)[17] found that serum miR-21, miR-122, and miR-223 were significantly elevated in patients with HCC compared with healthy controls, however they stated that since the significantly higher levels of serum miR-21 and miR-223 have been reported in patients with other types of human cancer compared with healthy controls, they may not be specific markers for HCC, nevertheless, miR-122 has been shown to be liver-specific.

Another study found that the levels of miR-122 in serum samples from HCC patients were significantly higher than healthy subjects and its level was significantly reduced in the post-operative serum samples when compared to the pre-operative samples[18].

MiR-122 not only is evolutionary conserved across species but also was identified as the most abundant liver specific miRNA constituting 70% of total hepatic miRNAs while cloning small RNAs from different tissues in mice[19]. miR-122 facilitates replication and translation of hepatitis C viral RNA and positively regulates cholesterol and triglyceride level[18]. For example, miR-122 binds to the 5' noncoding region of hepatitis C virus RNA, a highly conserved region in all six HCV genotypes, suggesting that miR-122 is an essential element of the HCV replication-adaptation to the liver. It has been shown

that functional inactivation of miR-122 leads to 80% reduction of HCV RNA replication, suggesting that loss of miR-122 in HCC may increase resistance of cancer cells to HCV replication. On the other hand, down-regulation of miR-122 was detected in more than 70% of HCC patients in a study done by Gramantier et al. (2007)[20]. It was shown that the level of miR-122 expression increases in the mouse liver throughout development, to reach the maximum just before birth. Thus, the loss of expression of miR-122 of HCC cells may represent either a differentiation reversion or a block to a less differentiated status of liver cells. In our study, it appears contrary and unexpected that the levels of miR-122 are elevated in serum of HCC patients. Other results showed that the elevated serum miR-122 is presented not only in HBV patients with HCC but also in HBV patients without HCC, suggesting that the elevated miR-122 in the serum of patients may also reflect liver injury[21]. Hepatocytes contain abundant miR-122 and damage of hepatocytes caused by inflammation due to virus infection or cancer would be expected to release significant amount of this miRNA into the circulation. Because serum miRNAs have been shown to be very stable[22], miRNAs leaked from damaged hepatocytes would accumulate in blood to a high level. This might explain why miR-122 is down-regulated in HCC tissues but elevated in serum of HBV patients without or with HCC[17]. miR-122 is suggested to play a bivalent role in hepatocarcinogenesis of HCV and non-HCV etiology[23] .

Comparing the results of measuring AFP and miR-122,it was found that AFP was normal in 18% of HCC patients while miR-122 was elevated in 100% of patients, in other words AFP sensitivity was 82% and specificity was 100%, while miR-122 sensitivity was 100% and specificity was 100%. This is in accordance with the recommendation of the American association for the study of liver diseases that AFP should not be used as a sole marker for HCC screening[24]. In addition, the

negative predictive value of AFP was only 66.67% while it was 100% for miR-122.

In conclusion, serum miR-122 might serve as a novel and potential biomarker for detection of HCC .Our data serves as a basis for further investigation, preferably in large prospective studies before miR-122 can be used as a noninvasive screening tool for HCC in routine clinical practice.

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