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RESEARCH ARTICLE

The role of MiRNA 122 and 494 as a Liquid Biopsy Markers for the Diagnostic Patients with the end Stages of Hepatocellular Carcinoma in Iraq

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ABSTRACT

Hepatocellular Carcinoma (HCC) Disease is the most popular and widespread kind of the liver cancer, It constitutes about 90% of occurrences; This disease has become a source of fear and an increasing danger to public health, as it ranks as the 4 largest cause of death worldwide at the moment. Evaluation of possibility of using miRNA122 and miRNA494 as predictive factors for the incidence of HCC disease in infected patients at the Oncology Center at the diwaniya Teaching Hospital and the Middle Euphrates Oncology Center in Najaf. Our present investigation was established in the Middle Euphrates Oncology Hospital and the Al-diwaniayh Oncology Center in Najaf. Sample collection took place between January and November of 2023. 44 individuals were selected as the patient group, and 44 uninfected people were chosen as the control group. The mean age of patients diagnosed with HCC was 65.13 ± 6.74 years, whereas in the control group was 60.70 ± 10.48 years. Based on these data, it was determined there is no difference in morale statistically in morality in both groups (P = 0.103). The study found that patients who suffer from HCC substantially reduced levels of miRNA-122 gene expression compared to control (0.102 ± 0.024 vs 1.16 ± 0.217, respectively, However, miRNA-494 gene expression was considerably greater in HCC patients compared to healthy controls (20.24 ± 2.56 vs 1.26 ± 0.88, respectively, P< 0.001). By using the ROC analysis , Our findings illustrate that both biomarkers that used in our current study it has a predictive value for the incidence of HCC and therefore it can be used as a liquid biopsy markers for the Diagnostic patients with for the detection or early diagnosis of the HCC .

INTRODUCTION

The liver is an organ possessing many unusual features, anatomical and functional, It is a large, solid, heavy glandular organ, located in the abdominal region of the human body (1). A prevalent disease of the digestive tract, liver cancer is caused by the growth and dissemination of abnormal cells inside the liver (2) (3). Liver cancer classified in the second place of cancer-related death and is the 5 most dangerous cancer in Asia (4). With a length of roughly 21–30 nucleotides, miRNAs are small, solitary, internal, non-coding microRNAs that have the power to control the expression of particular genes, or so-called target genes (5). It has been shown that the human genome encodes about 2600 species of miRNAs (6).

Around (70%) of the total miRNAs in liver tissue are hepato-specific miRNAs called miR-122, which has modulatory effects on liver disorders. (7),(8),(9) . Many studies revealed that miR-122 have tumor suppressor activity causing cancer cells to undergo apoptosis by stopping their cell cycle (10).(11),(12). It is interesting to use miR-122 as a marker for diagnosis as they are more stable and accurately measurable in various specimens by RT- PCR. Its assessment non-invasively makes it an interesting marker for variation in HCV replication and a potential biomarkers for cancer screening (13),(14) . A miRNA called miRNA 494 is found on chromosome 14q32.31 and is involved in carcinogenesis at different stages(15). (16) revealed that a subset of HCCs with stem cell characteristics had higher levels of miR-494, and they demonstrated how this increased expression affected sorafenib resistance through the energization the mTOR pathway. Through G6pc targeting also HIF-1A pathway activation, the miR-494 encourage the metabolic shift of HCC cells to the glycolytic phenotype. miR-494 / G6pc axis played an active role in the metabolic pliability For the cells of cancer, resulting in the cumulation of glycogen and lipid drops that favored cell survival under challenging environmental conditions (17). So, the development of novel serum indicators to aid in the early detection of HCC is the aim of this work.

METHODOLOGY

Collection of blood sample

With a 5 milliliter syringe. Five milliliters of blood were drawn from the control and patient groups. A volume of about 4 milliliters of blood was moved to anticoagulant-free tubes (Gel tubes), where it clotted for 2 min at 37°. The serum was separated by centrifugation for ten minutes at 3000 rpm. Using eppendorf tubes, the separated serum was distributed into portions and stored at (-80°C) for qPCR .

The serum total RNA extraction & qRT-PCR

As instructions by the manufacturer, the total RNA was isolated from the serum samples using a TRIzol® reagent kit. The Real-Time PCR technique was utilized to perform the RT-qPCR expression analysis and quantification of miRNA 122, miRNA 494, and the housekeeping gene (GAPDH) in patient and normal serum samples. The procedure followed the guidelines provided by (18).

Primers

The qPCR Primers for miRNA l22 (MIMAT0000421) and miR-494 (MIMAT0002816) were created for this work by selecting the miRNA sequence using The Sanger Center miRNA database Registry and by utilizing the miRNA Primer Design Tool. In contrast, the NCBI-Database and Primer3 plus design online were used in this investigation to create the qPCR Housekeeping gene (GAPDH) (NM_001256799.3). The following table shows the primers that (Macrogen business, Korea) as following table:

PRIMER		SEQUENCE (5'-3')
miRNA universal RT primers		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTT TTTTTTTTVN
miR-122 qPCR primer	F	AACAAGTGGAGTGTGACAATGG
	R	GTCGTATCCAGTGCAGGGT

miR-494 qPCR primer	F	AACAAGTGAAACATACACGGGAAA
	R	GTCGTATCCAGTGCAGGGT
GAPDH qPCR primer	F	AAAATCAAGTGGGGCGATGC
	R	TTCTCCATGGTGGTGAAGACG

Data analysis of qPCR

The quantitative assay gene expression levels "fold change" method (the ΔCT Method Using the reference Gene) was used to assess the q RT-PCR data results for both the target and housekeeping genes, as described by (19) as the subsequent equation:

$$\Delta\Delta CT = \Delta CT \text{ (Control)} - \Delta CT \text{ (Test)}$$
$$\text{Fold change} = 2\text{-}CT\ \Delta\Delta CT \text{ (target / HKG)}.$$

$$\Delta CT \text{ (Test)} = CT \text{ (target gene, test)} - CT \text{ (HKG gene, test)}$$

$$\Delta CT \text{ (Control)} = CT \text{ (target gene, control)} - CT \text{ (HKG gene, control)}$$

$$\Delta\Delta CT = \Delta CT \text{ (Test)} - \Delta CT \text{ (Control)}$$

$$\text{Fold change (target / HKG)} = 2^{-CT}\ \Delta\Delta CT.$$

Analytical statistics

The Microsoft office Excel 2010 , and the statistical program for the social sciences (spss version 26) had used to gather, compile, analyze, and present the data. After determining which variables were normally distributed and which weren't and running the Kolmogorov-Smirnov normality test, quantitative data were shown in the form of mean and standard deviation. The investigation of mean differences between any of the two groups was conducted using the independent sample(t-test), given that the mutable was typically distributed. Under the condition that the variable is normally distributed, the one-way (ANOVA) test was utilized for examine the mean differences among more than a couple groups. Any two categorical variables could be studied for association using the chi-square test. To quantify risk, the odds ratio and 95% confidence interval were determined. To identify the threshold value that indicates a favorable outcome, the recipient (20).

RESULTS

Features of the research population

Our current study included 44 patient's incidence with HCC and 44 unincidence people, table (1) shows the demographic features of both sick and control persons. The average age of patients with hepatocellular carcinoma was 65.13 ± 6.74 years, while that of control subjects were (60.70 ± 10.48) years. There was no significative difference amongst the groups (P = 0.103). Regarding to the sex or gender, in overall, 52 (59.1%) male and 36 (40.9%) female were included. Patients with hepatocellular carcinoma included 30 (68.2%) cases were male gender and 14 (31.8%)cases were female, while control subjects included 22 (50.0 %) cases were male gender and 22 (50.0 %) cases were female and There was no significant variation in frequency distribution between patients and

control subjects based on gender (P = 0.083). Sick group had the higher significant BMI (p<0.001) than the control group (23.81 ± 2.67 vs. 18.19 ± 1.52). The comparison of hematological parameters is also included in the next table:

Table 1: Distinguishing characteristic of 44 incidece with hepatocellular carcinoma and control

Feature	Patients N =**44**	Control N =4**4**	*P*
Age (years)	65.13 ± 6.74	60.70 ± 10.48	0.**103**
Sex			
Male	30 (68.2%)	22 (50.0 %)	0.**083**
Female	14 (31.8%)	22 (50.0%)	
BMI kg/m2	18.19 ± 1.52	2**3**.81 ± 2.67	< 0.001
RBC x10^6 uL	3.89 ± 0.53	4.34 ± 0.56	0.038
Hemoglobin (HGB) g/dL	9.62 ± 0.86	13.35 ± 0.96	< 0.001
WBC x103 g/L	13.29 ± 0.91	9.86 ± 0.74	< 0.001
Platelets x103g/L	63.39 ± 22.90	280.10 ± 27.89	< 0.001
Neutrophil x103 g/L	11.09 ± 0.34	2.83 ± 0.47	< 0.001
lymphocyte x103 g/L	0.69 ± 0.045	1.76 ± 0.74	< 0.001
NL ratio	16.01 ± 1.11	2.01 ±0.81	< 0.001

Family History of breast cancer

The presence of family history is a significant contributing factor in hepatocellular carcinoma. This study showed 16 (36.4%) of HCC patients have positive family history, and 28 (63.6%) of HCC patients have negative family history, figure 1.

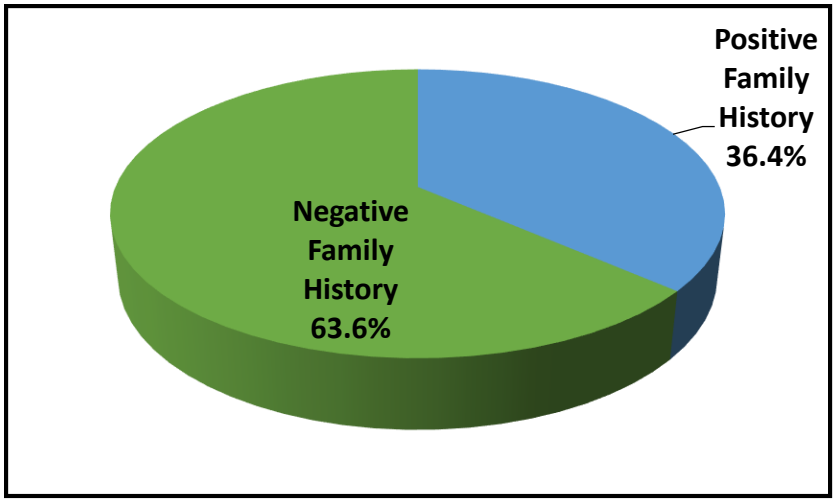

Figure 1: Distribution of HCC patients based on family history

Measurements of Gene expression parameters

Patients with HCC had significantly reduced mean miRNA-122 gene expression levels compare with control participants (0.102 ± 0.024 vs 1.16 ± 0.217, Sequentially, P< 0.001). However, miRNA-494 gene expression was considerably greater in HCC patients compared to healthy controls (20.24 ± 2.56 vs 1.26 ± 0.88, respectively, P< 0.001).

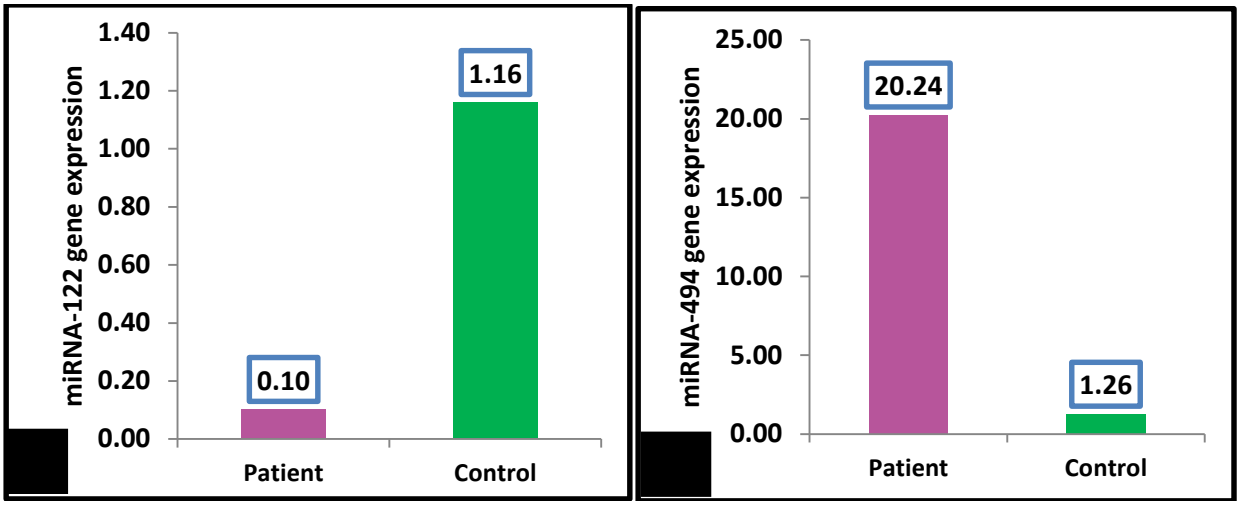

Figure 2: (A) *miRNA-122 gene expression* in patients incidence with HCC and control group. (B) *miRNA-494 gene expression* in patients incidence with the HCC Disease and control group. P < 0.05.

Diagnostically accuracy of Gene expression parameters

A receiver running feature (ROC) assaying was conducted to assessment the diagnostically accuracy to employing gene expression characteristics to identify HCC patients from healthy control subjects. An ideal miRNA-122 gene expression cut-off value of > 0.39-fold resulted in an AUC value of 1.000 (95% confidence interval [CI], 1.000-1.000, P < 0.001), sensitivity of 100.0%, particularity of 100.0%, PPV of 100.0%, and NPV of 100.0%. A Resistin cut-off value of more than 3.65 may accurately discriminate HCC patients from control, with a susceptibility of 100.0%, specificity of 100.0%, PPV of 100.0%, and NPV of 100.0%.

Table 2: Roc curve of Gene expression parameters

Characteristic	***miRNA-122***	***miRNA-494***
Cutoff value	< 0.39	**>** 3.65
P value	**< 0.001**	**< 0.001**
susceptibility%	100.0 %	100.0 %
particularity %	100.0%	100.0%
PPV %	100.0 %	100.0 %
NPV %	100.0%	100.0%
AUC (95% CI)	1.000 (1.000- 1.00)	1.000 (1.000- 1.00)

CI: Confidence interval, AUC : Area under curve.

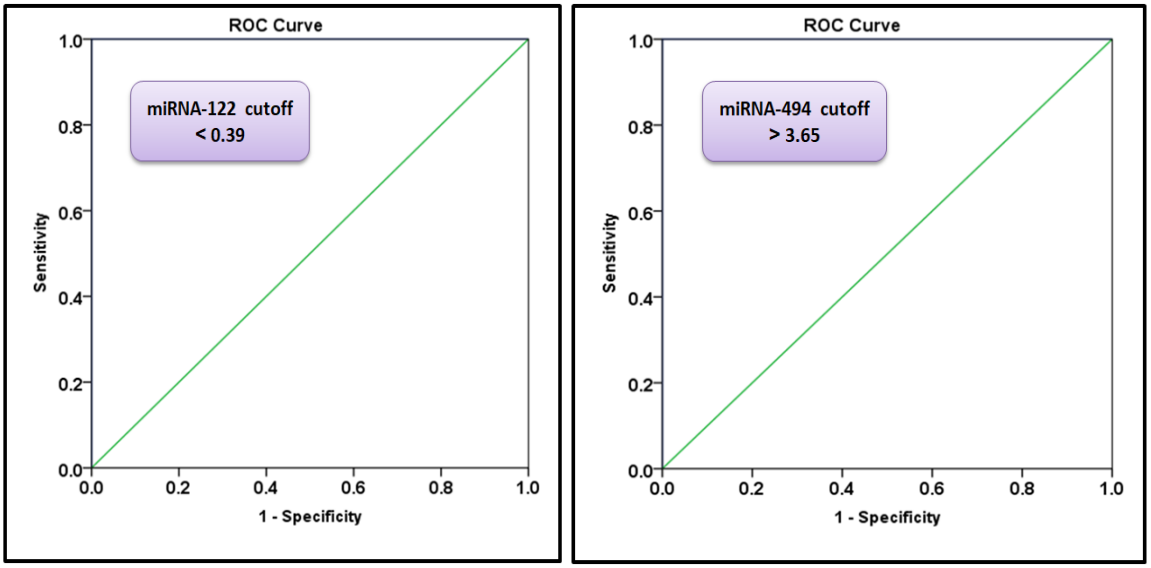

Figure 3: Receiver operator feature curve analyzation of gene expression parameters to determining a probable diagnostic cutoff value.

Correlation between *Gene expression parameters* and other parameters

The correlations between *gene expression parameters* and other parameters in patients with HCC were shown in tables (3). The present results show significant negative correlation between *miRNA-122 and miRNA-494 gene expression* (r = -0.501, p = 0.016), *miRNA122 gene expression* and Neutrophil count (r= -0.452, p= 0.030), *miRNA-122 gene expression* and Lymphocyte count (r = -0.472, p = 0.023), and *miRNA-494 gene expression* and HB (r= -0.513, p= 0.001), But the present results show non-significant correlation between all other parameters.

Table (3): Correlation between Gene expression parameters and other parameters.

Characteristic	*Gene expression parameters*			
	miRNA-122		***miRNA-494***	
	R	***P***	***r***	***P***
miRNA-122	1		-0.501	0.001
miRNA-494	-0.501	0.001	1	
RBC	0.072	0.744	-0.229	0.292
HB	0.024	0.913	-0.507	0.001*
WBC	-0.019	0.931	-0.272	0.207
Neutrophil	-0.452	0.030*	0.272	0.210
Lymphocyte	-0.472	0.023*	0.298	0.167
Platelets	-0.123	0.576	-0.174	0.427

r: correlation coefficient

DISCUSSION

(21) mentioned that the examination of the average fold change of the expressiveness level of miRNA122 in patients sera compare with the control group revealed the small fold reduce in expression in the all analyzed collectives, an upper level were reported in the HCC patients with a median fold change (0.8) compared to the control group. .

In order to test the benefit of the miR-122 as a pointer for diagnosing cirrhosis in chronic infestation with hepatitis C virus and like a diagnostic method for early discover of HCC, our findings revealed the significantlly decrease with serum miR-122 in HCC patients compare with CHC patients and controls (22) .

(23) noted that the miRNAs are essential Organizations for several intracellular Operations connected to medication resistance; for example, MicroRNA 494 was upregulated and involved in sorafenib-induced resistance.

(24) To examine the suppressive effect of the miR 494 in the LSCC (lung squamous cell carcinoma) progression and explain its regulatory mechanism, so that the expressiveness level of miR 494 in the LSCC tissues were greatly greater than those in the surrounding non-cancer tissues. furthermore, microRNA 494 levels was measured in 4 LSCC cell lines and "16 HBE", whereby served like a control. MiR 494 expression was increased in all LSCC cells compared to 16HBE.

(17)The miRNA-494 Stimulates the metabolic shift of the hepatocellular carcinoma cells to the glycolytic phenotype via (G6pc) targeting and (HIF-1A) pathway energization or activation, with the goal of proving its involvement in HCC metabolic reprogramming, identifying new miRNA based on therapeutics combinatives, additionally evaluating miR494 can be used as the circulating biomarker. The miR494 /G6pc axis was active in cancer cells' metabolic plasticity, resulting the cumulating of glycogen and lipid drops, who enhanced cell survivability in adverse environmental circumstances. Preclinical models and a preliminarily cohort of hepatocellular carcinoma patients Explained that the increase in miR494 serum levels were related with sorafenib resistivity.

CONCLUSIONS

The miRNA-122 cutoff value was greater than 0.39 fold, with sensitivity, specificity , positive prognostic value (PPV), negative prognostic value (NPV), and area under curve values (AUV) of 100.0%, 100.0%, 100.0%, & 1.000 (1.000-1.000). The miRNA-494 cut off value was < 3.65-fold with sensitivity, specificity, PPV, NPV, and area under curve of 100.0%, 100.0%, 100.0%, and 1.000 (1.000-1.000). The current results reveal that miRNA-122 and miRNA-494 are strong diagnostic markers.

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