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ORIGINAL ARTICLE

Evaluation of Circulating MicroRNA 483-5p as a Useful Diagnostic Tool of Hepatocellular Carcinoma in Egyptian Patients

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ABSTRACT

Background: *HCC comes in the sixth rank in all tumors that influence the human body and it is generally connected with interminable liver cirrhosis in 80% of patients. In Egypt, the rate of HCC has expanded pointedly in the last decade. It is considered as a heterogeneous illness including variable types of neoplasms, which includes distinctive profile changes in both mRNA and miRNA expression. One of them is miRNA 483-5P which was found to be up-regulated in hepatocelleular carcinoma.* ***Objectives:*** *To assess the diagnostic and prognostic potential of circulating miRNA 483-5p in HCC in Egyptian patients in comparison to chronic liver disease patients and healthy controls.* ***Methodology:*** *This study was done on 100 HCC patients, 50 chronic liver disease (CLD) due to HCV infection and 50 healthy subjects. Serum alpha fetoprotein (AFP) was measured in all subjects by Chemiluminescence. The relative quantitation of miRNA 483-5p was determined by real-time quantitative Polymerase Chain Reaction (RT-qPCR).* ***Results:*** *the results revealed an over expression of miRNA-483-5p in HCC group ($p < 0.001$) compared to both CLD and healthy subjects, while no significant change was detected between CLD and healthy subjects. MiRNA483-5p expression increased significantly with increased number of focal lesions and size of tumor. Receiver Operator of Characteristics (ROC) curve analysis of plasma miRNA-483-5p revealed that, at a cut-off value of 3. 22 (fold expression), the sensitivity and specificity for differentiation of HCC cases were 88.0% and 92.0%, respectively.* ***Conclusion:*** *Circulating miRNA483-5p can be used as an early diagnostic biomarker for detection of HCC.*

INTRODUCTION

Hepatocellular Carcinoma (HCC) is the sixth most essential tumor worldwide to the extent number of cases and the second genuine promoter to malignancy mortality in man. The survival rates in the United States and made countries are only 3% to 5%[1].

Hepatocellular carcinoma (HCC) is the sixth most regular harmful tumor and the third most basic reason for disease demise worldwide[2].

In Egypt, the rate of HCC has extended firmly in the latest decade. Up till now no reasonable biomarkers for the early confirmation and figure of HCC. By surgical resection and by liver transplantation, just around 30% to 40% of HCC patients can get effective treatment at the helpful time[3].

MicroRNAs (miRNAs) are single-stranded, noncoding RNAs of 20- 23 nt long. They have significant roles in numerous cellular processes by bonding to messenger RNAs (mRNAs) and reducing their stability. They play part in various cell normal strategies, including embryonic headway, cell division, and tumorigenesis[4].

MicroRNAs can work both as tumor silencers and as oncogenes [5]. As tumor silencers, they quell oncogenic targets, however they are normally down-directed in growth tissues[6]. Others are up-controlled and have an invigorating part for tumor movement[7]. These miRNAs can control many cancer hallmarks through stimulation of unusual pathways and biological processes (adhesion, proliferation, transcription, translation and inflammation), hence, several cancer hallmarks that contribute to cancer beginning and growth are affected [8]. This

double part as oncomiRNAs and tumor silencers has empowered various reviews on miRNAs and cancers, prompting full identifications of miRNA target genes[9].

MicroRNAs manage post-transcriptional quality expression, by official to the 3-untranslated area (3-UTR) of particular target dispatcher RNAs (mRNAs), which thusly causes mRNA corruption or translational suppression[10]. MiR-483 gene is arranged on genomic chromosome at 11p15.5 in the second intron of Insulin-like Growth Factor 2 (Igf2) and produces two create assorted structures: miR-483–3p and miR-483–5p, differentially incorporated into liver pathologies [11].

MiR-483-5p and miR-483-3p were perceived from a human embryonic liver. Reports have recommended that some intragenic miRNAs co-express and work together with their host qualities, however that diverse miRNAs don't. Igf2 over-expression propels augmentation and carcinogenesis in the midst of the development from liver fibrosis to HCC. Furthermore, miR-483 is up-overseen in around half of human tumors including adrenocortical carcinoma and HCC, and its oncogenic targets (PUMA, CTNNB1, IGF1R) have been perceived [12].

The aim of this work is to evaluate the diagnostic and prognostic potential of circulating miRNA-483-5p in HCC in Egyptian patients.

METHODOLOGY

Study population:

This study was done on 100 newly diagnosed untreated HCC patients and 50 patients with chronic liver disease (CLD) because of HCV disease admitted to Inpatients Wards and Outpatient Center of Hepatology branch of National Liver Institute, Menoufia University, in the period from January 2016 to December 2016. In addition to the previous groups, 50 age and sex matched apparently healthy subjects served as a control group. The diagnosis of HCC was based on clinical examination, laboratory tests, ultrasonography and spiral CT. CLD patients were diagnosed by laboratory tests and ultrasonography. All the participants were subjected to full history taking, complete clinical examination, abdominal ultra-sonography and/or CT.

Laboratory investigations:

Ten ml venous blood samples were collected from all participants and divided into three parts. The first part was collected in plain tube and used for routine laboratory investigations, including liver function tests using fully automated auto 111 analyzer SYNCHRON CX9ALX (Beckman Coulter Inc., CA, USA) and serum AFP concentration was measured using the Automated Chemiluminescence System (ACS: 180 provided by Siemens Medical Solutions Diagnostics Corporation, USA). The second part was collected in an ethylene diamine tetra acetic acid (EDTA) containing tube which used for CBC assessment using Sysmex K-21, (Sysmex Corporation, Kobe, Japan)..The third part was collected in an EDTA containing tube and used immediately for RNA - miRNA extraction and molecular testing.

Molecular testing:

Real time PCR technology (using 7500 fast real time PCR – TaqMan microRNA and RNA Control Assay) was used for assessments of miRNA-483-5p and its control gene (RNU1).

Extraction and cDNA synthesis:

miRNA extraction and quantification:

RNA was extracted from fresh EDTA treated blood sample using PureLink RNA Mini Kit (Ambion, Life Technology) and QIAzol (Lysis solution) regarding the manufacturer's protocol.

Isolation of miRNAs from blood followed the protocol for miRN easy RNA isolation kit (Qiagen, Germany). Separation of plasma took place immediately within 2hours from blood sample collection. The extracted total RNA were subjected to Reverse transcription.

RT-PCR:

Single-stranded cDNAs were created utilizing TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) by means of blending RT Primer (3 µL), 10X RT Buffer (1.5µL), RNase Inhibitor (0.19 µL), dNTPs blend (0.15 µL), MultiScribeTM Reverse Transcriptase (1 µlL), and nuclease free water (4.16 µL). RNA tests (5µL) were included, blended and quickly centrifuged with the past arranged parts. The programming of warm cycler condition was as the followings: hold for 30 min at 16 ^{0}**C**, hold for 30 min at 42 ^{0}**C**, then, end of the response by warming at 85 ^{0}**C** for 5 min.

Amplification:

Determination of miRNA483-5p levels was done by TaqManmiRNA Assay using Universal TaqMan master mix (Applied Biosystems, Thermo Fisher Scientific) regarding the manufacturer's protocol. Fluorescence measurements were made in every cycle (Fig 3, 4)

and the cycling conditions used for amplification of miRNA were: initial denaturation step at 95 ^{0}**C** for 10 min followed by 40 cycles of 95 ^{0}**C** for 15 s and 40 cycles of 60 ^{0}**C** for 60 s.The primers of The RNU1(mi RNA 16) and miRNA-483-5p were supplied by QIAGEN, GERMANY):

The *Internal Control* (The RNU1 (mi RNA 16) *Sequence:*

GAACTTATTGACGGGCGGACAGAAACTGTGTG CTGATTGTCACGTTCTGATT *and*

miRNA-483-5p probe sequence:

AAGACGGGAGGAAAGAAGGGAG

Quantification:

Quantification of miRNA expression was achieved by measuring the fractional cycle number at which the measure of expression achieved a fixed threshold (Ct), which was directly related to the amount of the product.

The relative quantification given by the Ct vaues was resolved and the control gene Ct subtracted to accomplish ΔCt ΔCt = Ct (gene of interest) – Ct (control gene).

At that point, the relative expression level was resolved as $2^{-\Delta\Delta Ct}$, (where ΔΔ Ct = ΔCt (target test)- ΔCt (reference test).

Statistical analysis:

Data were collected and analyzed by IBM SPSS (Statistical Package for the sociology) version 23.0 (SPSS, Inc, Chicago, IL, USA, 2015). ANOVA, ANOVA test was used for comparison of quantitative variables between more than two groups of normally distributed data with Tuckey test as post Hoc test while; Kruskal Wallis test was used for comparison of quantitative variables between more than two groups of not normal distributed data with Tamhane's test as post hoc test. Receiver operator characteristic (ROC) with respective points of maximal accuracy for sensitivity and specificity were generated to determine biomarker performance

RESULTS

The overall 200 subjects that included in this study, 100 HCC patients with mean age of 53.66 ± 7.76 years, consisted of 72 males and 28 females. Fifty CLD patients with mean age of 50.28 ± 4.63 years, consisted of 28 males and 22 females. Another 50 healthy volunteers with mean age of 51.24 ± 5.33 years consisted of 26 males and 24 females. The studied groups were homogenous regarding age and sex ($p > 0.05$).

Comparison between the 3 studied groups regarding mean relative quantities (RQs) of miRNA483-5p expression revealed that there was significant increase in miRNA483-5p in HCC group compared to each of CLD and control groups ($p < 0.001$). However, there was no significant difference between CLD and control groups ($p > 0.05$) (Table 1 and figure 1). The mean RQ of miRNA483-5p showed significant increase with presence of cirrhosis, increased number of focal lesions and larger size of the tumor (table 2).

Table 1: Comparison between the 3 studied groups regarding the mean of relative quantity (RQ) ofcirculating miRNA-483:

Studied variables	HCC (n=100) Mean ±SD	CLD (n=50) Mean ±SD	Control (n=50) Mean ±SD	Kruskal Wallis test	P value	Post Hoc
miRNA-483 RQ (Folds):	29.15 ± 25.79	2.70 ± 4.14	1.44 ± 1.94	57.72	<0.001	P1 <0.001 P2 <0.001 P3 >0.05

Table 2: Comparison between some tumor characteristics regarding mean RQ of miRNA-483 in HCC group:

Pathological Criteria	miRNA-483 RQ	Mann Whitney test	P value
No of lesions: **Single (n=10):** **Multiple (n=40):**	 6.55± 6.18 34.81 ± 25.75	3.82	<0.001
Size of the tumors: **< 3 cm (n=6):** **> 3 cm (n=44):**	 6.39 ± 7.99 32.26 ± 25.85	 2.86	 <0.01

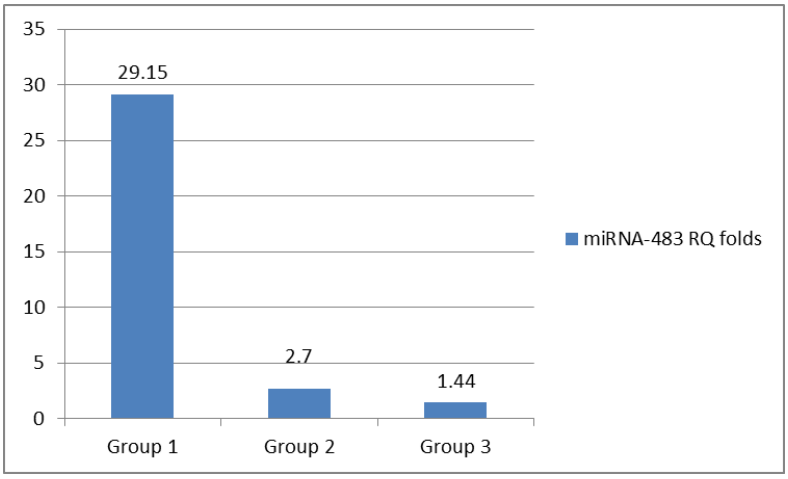

Fig. 1: Mean relative quantity of circulating miRNA-483 in studied groups.

Receiver Operator Characteristics (ROC) examination for distinction of HCC cases from those without HCC using AFP and miRNA483-5p showed that: For miRNA-483the best cutoff was 3.22 RQ with sensitivity, specificity, PPV, NPV and overall accuracy of 88.0%, 92.0%, 92.0%, 88.0% and 90.0% respectively, while the best cut-off of serum AFP was 92.5 ng/ml with 80.0 % sensitivity, 90.0% specificity, 89.0% PPV, 82.0% NPV and 85.0% overall accuracy table 3 and figure 2 .

Table 3: Receiver Operator of Characteristics (ROC) curve and cut off point of RQ and AFP between HCC and non HCC:

Marker	AUC	Cut off point	Sensitivity	Specificity	PPV	NPV	Accuracy
RQ of gene	0.92	3.22	88.0%	92.0%	92.0%	88.0%	90.0%
AFP	0.92	92.5	80.0%	90.0%	89.0%	82.0%	85.0%

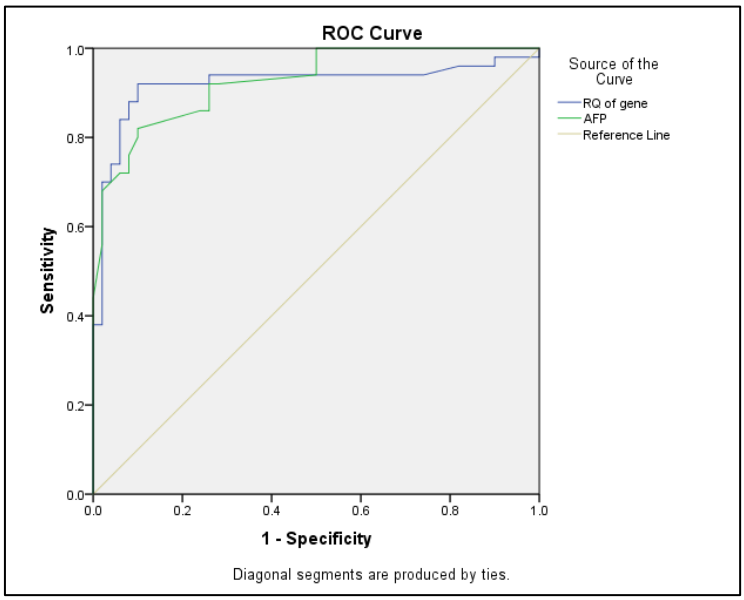

Fig. 2: ROC curve of both RQ of gene and AFP.

Table 4 shows that:

- There was non significant difference between HCC and CLD groups regarding all studied variables except ALP, GGT and AFP which showed significant increase in HCC group.
- There was significant increase of all studied parameters except levels of TP and Alb in HCC group compared to control group.
- There was a significant increase of the mean serum level of AST, ALT, ALP, GGT, TB, DB and INR, and significant decrease of the mean serum level of TP and Alb in CLD group compared to control, meanwhile no significant difference was detected between the two groups regarding AFP.
- AFP was significantly higher in HCC group compared to both CLD and control group.
- INR was significantly higher in HCC group than both CLD and control groups.

Table 4: Comparison between the 3 studied groups regarding liver function tests, AFP and INR:

Studied variables	HCC (n=100) Mean ±SD	CLD (n=50) Mean ±SD	Control (n=50) Mean ±SD	ANOVA	P value	Post Hoc
AST: (IU/L)	57.96 ± 56.85	50.48 ± 23.58	19.08 ± 3.78	13.77*	0.001	P1 >0.05 P2< 0.001 P3 <0.001
ALT: (IU/L)	60.86 ± 33.04	47.84 ±22.45	18.24 ± 6.56	50.48*	<0.0001	P1 >0.05 P2 <0.001 P3 <0.001
ALP: (IU/L)	121.87 ± 39.89	100.08 ± 30.74	61.00 ± 13.11	28.59	<0.0001	P1< 0.01 P2 <0.001 P3 <0.001
GGT: (IU/L)	82.15 ± 45.88	43.70 ± 21.84	20.77 ± 8.58	45.89*	<0.0001	P1 <0.001 P2 <0.001 P3 <0.001
Bil. T: (mg/dl)	3.26 ± 1.76	2.06 ± 1.76	0.53 ± 0.18	54.27*	<0.0001	P1 <0.01 P2 <0.001 P3 <0.001
Bil. D: (mg/dl)	1.45 ± 0.89	1.11 ± 1.28	0.12 ± 0.04	53.47*	<0.0001	P1 >0.05 P2 <0.001 P3 <0.001
Albumin: (g/dl)	2.22 ± 0.65	2.76 ± 0.65	4.32 ± 0.41	83.63	<0.0001	P1 <0.01 P2 <0.001 P3 <0.001
TP: (g/dl)	6.42 ± 0.68	6.76 ± 0.81	7.43 ± 0.40	13.67	<0.0001	P1 >0.05 P2 <0.001 P3 <0.001
AFP: (ng/mL)	103.81 ± 27.50	50.76 ± 32.44	4.92 ±2.35	59.38	<0.001	P1 <0.001 P2 <0.001 P3 0.01
INR:	1.79 ± 0.63	1.52 ± 0.51	1.02 ± 0.02	54.78*	<0.0001	P1 >0.05 P2 <0.001 P3<0.001

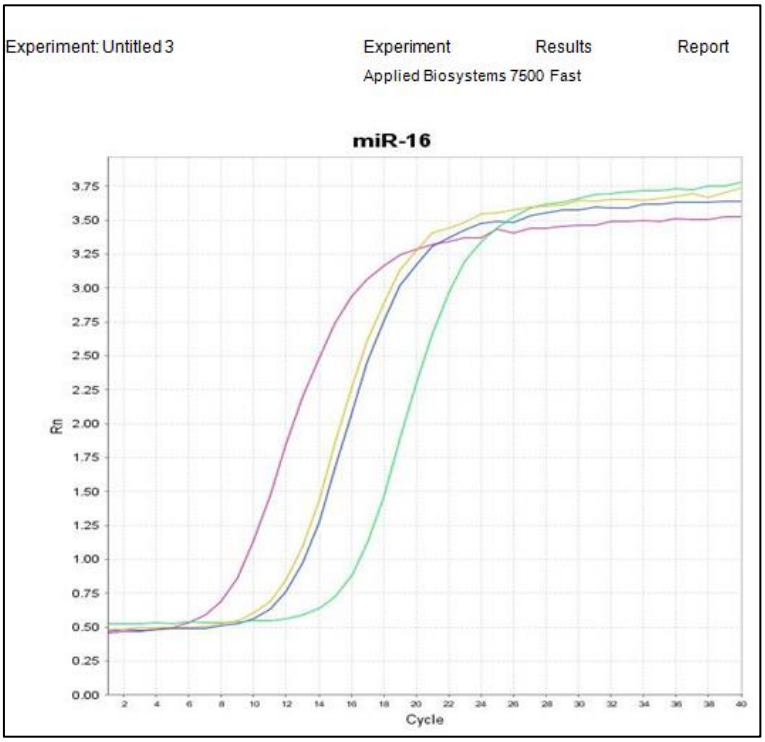

Fig. 3: Amplification plot of RNU1(miRNA 16)

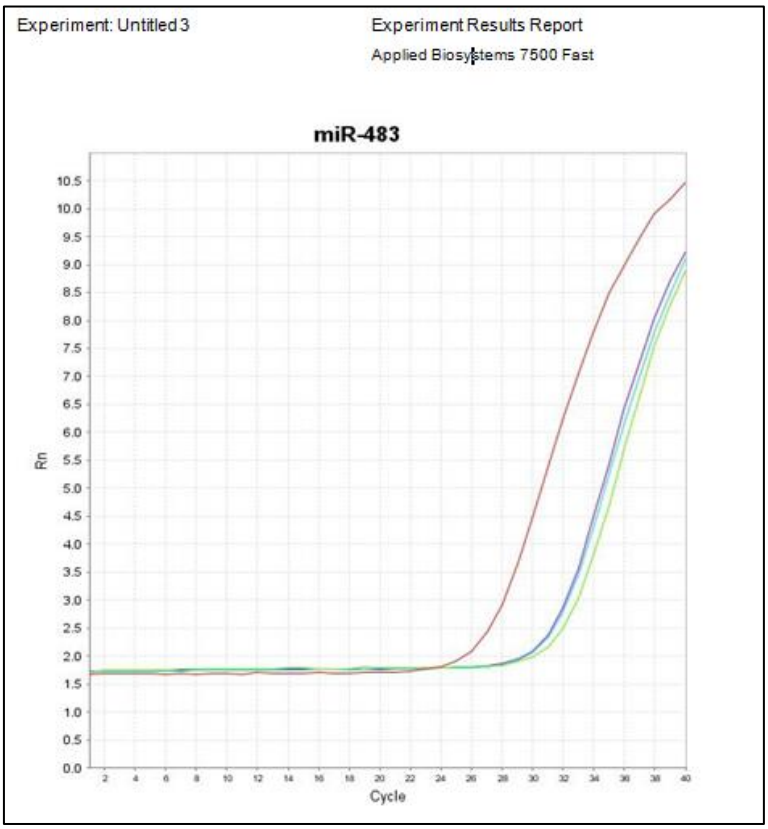

Fig. (4): Amplification plot of miRNA-483-5p

DISCUSSION

There is a long way to go in fighting against HCC and molecular targeted therapies are still needed to be further verified in clinical application. many evidences have documented that miRNAs are also concerned in oncogene and tumor suppressor pathways [13].

Serum alpha fetoprotein (AFP) measured in clinically accessible specimens has for quite some time been utilized as an early symptomatic biomarker of hepatocellular carcinoma, yet its sensitivity (39%–65%) and specificity (76%–94%) are poor [14,15].

miRNAs in liver, wear different expression profiles from non diseased livers to that among the patients with HCC including those with cirrhosis and hepatitis infection[16,17,18].

MicroRNA 483-5p is a 22-nucleotide (AAGACGGGAGGAAA GAAGGGAG) intronic develop microRNA .The MiR-483-5p goes about as an oncogene co-communicated with Igf2gene .it`s area in intron 2 of the Igf2 gene, and its objective qualities are Socs3 and Tnf-an .lso, we found that miR-483-5p was very communicated in liver, mind, heart, and kidney. This miRNA has additionally been accounted for to be up-regulated in other dangerous tumors The human miRNA483-5p gene is situated on in addition to strand of chromosome 11also known as MIRN483; mir-483; hsa-mir-483.[19].

HCC as other malignancies is attributed to accumulated genetic alterations. As an oncomir, miRNA-483-5p is up-regulated in a variety of human malignancies. Overexpression of miR-21 promoted proliferation and protected against apoptosis in various tumors (e.g., breast, lung, colon, and liver cancers [20].

Distorted miRNA expression has been related with an assortment of malignancies, including hepatocellular carcinoma by analyzing tumor and non-tumor tissues. Several miRNAs with oncogenic qualities attributes are fundamentally up-regulated in hepatocellular carcinoma tumor tissues contrasted and non-tumor tissues, for example, the miRNAs (miR)- 17- , miR-21, miR-181b, miR-221, miR-222.[21].

Consistent with the fore mentioned studies, this work demonstrated that miRNA-483-5p is up-regulated in HCC where mean circulating miRNA-21 RQs in patients with HCC was significantly higher ($p < 0.001$) compared to that of patients with CLD and healthy individuals groups, which was also in agreement with other researchers as Zhoujing et al. and Jing al. [19,22]

MiR483-5P levels were significantly increased as potential prognostic marker in HCC and also accepted with Ma et al. [23].

In the current study, a significant increase ($p < 0.001$) in the mean RQ of miRNA-483-5p was observed with cirrhosis and progression of HCC, as it showed a significant increase in multiple focal lesions and larger size of tumor (>3cm) which indicate that circulating miRNA-483-5p could be a potential prognostic marker in HCC. Meanwhile, no significant change of the mean serum AFP levels was observed regarding the same characteristics of HCC tumors.

These results are consistent with the study done by Zhoujing et al. [19] who revealed that circulating miRNA-483-5p levels were significantly higher in advanced HCC compared with early HCC groups, but serum AFP level was not changed until tumor appearance.

For miRNA-483 the best cutoff was 3.22 (fold), RQ gave 88.0% sensitivity, 92.0% specificity, 92.0% PPV, 88.0%NPV and 90.0% overall accuracy with 0.92 AUC. These results make miRNA-483 superior to serum AFP for differentiation of HCC cases from those without HCC. At cutoff point 92.5 ng/ml, AFP had 80.0%sensitivity, 90.0% specificity, 89.0% PPV, 82.0% NPV and 85.0% overall accuracy.

Jing al.[22] who demonstrated that ROC curve analysis of circulating miRNA-483-5p the sensitivity and specificity were, respectively, 75.5% and 89.8%. controls with an area under the curve of 0.908 ($P < 0.0001$).

CONCLUSION

For differentiating HCC patients from healthy adults miRNA-483-5p could be a promising biomarker of HCC. Furthermore, combined use of serum AFP and circulating miRNA-483-5p for detection of HCC cases, had an advantage over the use of AFP alone.

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