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Serum Expression Levels of miR-141 and miR-215 for Differentiation between Liver Cirrhosis, Chronic Hepatitis C and Hepatocellular Carcinoma Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Author SAMA designed the study, performed the laboratory work, managed the literature searches, managed the analyses of the results and wrote the first draft of the manuscript. Author ZZA wrote the protocol, participated in the designing of the study, participated in the performing the laboratory work and reviewed of the manuscript. Author HAY participated in the designing of the study, recruited the cases, performed the statistical analysis and reviewed of the manuscript. Author AMA managed the literature searches, recruited the cases, shared in analyses of the results and reviewed of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: To evaluate the ability of estimation of serum expression levels of micro RNA (miR-141 and miR-215 to differentiate between liver cirrhosis, chronic hepatitis C (CHC) and hepatocellular carcinoma (HCC) patients.

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Patients and Methods: The study included 25 liver cirrhosis patients, 25 CHC patients, 25 HCC patients and 15 volunteers (Control group). All patients underwent clinical examination, preliminary investigations and radiological workup. Fasting blood samples were withdrawn from all patients and controls for estimation of serum levels of α-fetoprotein (AFP), quantitative PCR estimation of HCV RNA titers and real-time PCR quantitation of serum expression levels of miR-215 and miR-215 -141.
Results: Serum AFP levels were significantly higher in HCC patients than cirrhosis and CHC patients. Estimated serum expression levels of miR-215 were significantly higher in CHC and HCC patients compared to both controls and cirrhosis, while serum expression levels of miR-141 were significantly lower in HCC patients compared to controls and cirrhosis patients and in CHC patients than controls. Estimated HCV viral load in CHC patients showed positive significant correlation with serum expression level of miR-215, while showed non-significant correlation with miR-141. Estimated serum levels of miR-215 and miR-141 could differentiate hepatic patients and controls with AUC= 0.872 and 0.250, respectively. Whereas, estimated serum levels of miR-215 could differentiate between cirrhosis and CHC patients with AUC= 0.899. Estimated serum levels of miR-215 and miR-141 also could be used to identify HCC patients out of hepatic disease patients with AUC of 0.818 and 0.351, respectively.
Conclusion: Serum expression levels of miR-215 and miR-141 could be used to identify hepatic disease patients with high positive predictive value (PPV) especially miR-215. miR-215 can differentiate between cirrhosis and CHC patients and correlated with HCV load. Serum levels of both miRs could assure diagnosis of HCC with high PPV.

Keywords: Hepatic diseases; miR-215; miR-141; HCC patients detection.

1. INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major health problem worldwide [1] and is a major risk factor for the development of hepatocellular carcinoma (HCC) [2]. However, the best means for evaluating liver impairment is still a major clinical challenge [3] and the diagnosis of cirrhosis portends an increased risk of morbidity and mortality [4].

Liver biopsy despite of being the gold standard for diagnosis of cirrhosis and staging of fibrosis (4) and to assess histological activity of HCV [5], it is an invasive procedure and has been associated with sampling error mostly due to suboptimal biopsy size [3]. Noninvasive testing has been shown to be equally predictive in ruling out or ruling in advanced fibrosis [6] and are becoming standard of care, which significantly reduces the need for liver biopsy [7].

Hepatocellular carcinoma is one of the most prevalent malignancies in the world [8]. HCC is one of the leading causes of cancer deaths and its current modes of treatment still palliative [9]. Diagnostic ability of estimated serum levels of liver enzymes is limited, but could be used to evaluate the response to treatment [10].

Micro RNAs (MiRs) are a class of endogenous small non-coding single-strand RNA molecules [11] that regulate gene expression at the post-transcriptional level [12]. In humans, more than 2000 mature MiRs have been identified [13].and affect the pathophysiology of internal diseases and can also reflect their presence [14].

Circulating MiRs are MiRs present in extracellular space including all body fluids and are released into the circulation either after the cell necrosis or by active transport [15]. Circulating miRs are stable with constant levels among the individuals of one species, methods determining their levels are reproducible and their levels differ between healthy and diseased individuals [14]. Changes of circulating miRs level occur in early oncogenesis, so could be a potential biomarkers for early detection of cancer [16].

The aim of the study was to evaluate the ability of estimation of the serum expression levels of miR-141 and miR-215 to differentiate between liver diseases especially cirrhosis and CHC which are the precursors of HCC [17,18,19].

2. PATIENTS AND METHODS

The current study was conducted at Departments of Tropical Medicine and Microriology, at Alansar and Saudi German Hospital, Saudia Arabia since June 2015 till Dec 2016. The study protocol was approved by Local Ethical Committee. All enrolled patients signed written fully informed consent for study participation and undergoing assigned investigations.

Table 1. Criteria and score points for calculation of Child-Pugh score [20]

Criteria	Score (points)		
	1	2	3
Serum total bilirubin	<2 mg/dl	2-3 mg/dl	>3 mg/dl
Serum albumin	>3.5 g/dl	2.8-3.5 g/dl	<2.8 g/dl
International normalized ratio (INR)	<1.7	1.71-2.20	>2.2
Ascites	No	Present	Tense
Encephalopathy	No	Grade I or II	Grade III or IV

Table 2. Primers for amplification of miR-215 and miR-141 expression

Primer	Sequence	REF
MiR-215	5'-ATG ACC TAT GAA TTG ACA GAC AA-3' (5'-3'sequence forward) 5'-GCT GTC AAC GAT ACG CTA CGT-3'5'-3' (5'-3'sequence reverse)	25
MiR-141	5'-CTG CCT TTA CAG CAA GAT-3' (5'-3'sequence forward) 5'-TTT ATT TCA TGC TCC CAA GGC GGG-3' (5'-3'sequence reverse)	26
U6	5'- CGC TTC GGC AGC ACA TAT AC-3' (5'-3' sequence forward) 5'-TTC ACG AAT TTG CGT GTC AT-3' (5'-3' sequence reverse).	25

2.1 Preliminary Evaluation

Demographic data included age, gender and body mass index (BMI) data.Routine liver function tests including estimation of serum alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TB), albumin and calculation of international normalized ratio (INR). Screening for hepatitis (HBsAg and HCV antibodies) was done using commercially available kits (Bio-RADFrance) with PEB III (Dade Behring).Abdominal ultrasonography (US) for hepatic scanning and evaluation of presence and/or severity of ascites and Abdominal CT or MRI imaging were done for assurance of inclusion and exclusion criteria.

2.2 Inclusion Criteria and Grouping

1. Cirrhosis group: Clinically cirrhosis was defined by clinical development of esophageal varices, splenomegaly or small liver size with irregular liver surface on imaging studies. Cirrhotic patients were classified according to Child-Pugh scoring system, as shown in above Table 1, as Class A with total score of 5-6 indicating mild liver disease, Class B with total score of 7-9 indicating moderate liver disease and Class C with total score of 10-15 indicating severe liver disease [20]. Only patients of Class A with total score of <7 were enrolled in the study.
2. Chronic hepatitis C (CHC) group: CHC infection was defined as hepatitis infection persisting for longer than 6 months with HCV antibody positive, increased serum ALT values and radiological assessment assured absence of any evidence of cancer.
3. Hepatocellular carcinoma (HCC) group: HCC diagnosis depended on biopsy confirmed histological examination, or by either of CT or MRI imaging in patients with serum α-fetoprotein (AFP) level ≥400 ng/ml [21].
4. Control group: Fifteen volunteers collected from those attending hospital blood banks after passing pre-blood donation investigations were enrolled as controls for laboratory investigations after exclusion of criteria for patients' inclusion criteria for other groups.

2.3 Exclusion Criteria

Exclusion criteria included HBV co-infection, associated biliary diseases, possibility of extra-hepatic diseases or malignancy, presence of other possible viral infections, current anti-viral therapy or advanced HCC.

2.4 Investigations

Fasting blood samples were withdrawn from all patients and controls and were divided into three parts:

1- The 1st part (3 ml) of the freshly drawn whole blood specimens was stored at 2-

25°C for no more than 6 hours prior to centrifugation. Plasma were separated from whole blood within 6 hours of collection by centrifugation at 800-1600 x g for 20 minutes at room temperature. HCV RNA titers were quantitatively determined by real-time polymerase chain reaction (PCR) using COBAS TaqMan HCV quantitative test, version 2.0 (Roche Molecular Systems, Inc., Branchburg, NJ, USA) with a linear range from 43 to 69,000,000 IU/ml according to manufacturer instructions [22]. Then, the $\log_{10}$ HCV RNA was calculated and considering the $\log_{10}$ value of 6 as cutoff log titer for HCV RNA levels corresponding to 10^6 IU/ml, patients were categorized as high serum viral load if the $\log_{10}$ titer was > 6 log titer of HCV RNA IU/ml and low serum viral load if the $\log_{10}$ H titer was ≤6 log titer of HCV RNA IU/ml [23].

2- The 2nd part of the freshly drawn blood sample was collected in plain tube and allowed to clot and centrifuged at 5000 rpm for 10 minutes and serum was collected and stored at -80°C till assayed for estimation of serum levels of AFP using the commercially available immunometric assay (Architect AFP assay, Abbott Laboratories, North Chicago, IL, USA) [24].

3- The 3rd part of the freshly drawn whole blood specimens were stored at 2-25°C for up to 24 hours followed by centrifugation to separate the serum, which was stored at -80°C until used for molecular assay of miRs according to manufacturer's instructions. Briefly, RNA isolation and quantification of miR-215 and miR-141 is as following:

- Total micro RNA was extracted from serum samples using RNeasy Protect Animal Blood kits (Qiagen kits) according to manufacture instructions. Two-step RT-PCR for miR-215 and miR-141 was performed using Qiagen miScript preAMP RT-PCR kit (Qiagen GmbH Hilden, Germany) for conversion of MiR to cDNA in a G-storm thermocycler (UK). Then, amplification and quantification of miR-215 and miR-141 was done by real time PCR in ABI 7900 (Applied Biosystem, USA) using SuperReal Premix Plus Quanti Tect. Kit, SYBR Green (Tiagen, Shanghai) according to manufacture's instructions and using the specific primers for each.
- Real time cycler conditions were:
  - For miR-215: Initial denaturation at 95°C for 15 min , followed by 45 amplification cycles of 95°C for 5 sec for denaturation, 58°C for 20 sec for annealing and 72°C for 30 sec for extension step.
  - For miR-141: Initial denaturation at 95°C for 5 min , followed by 45 amplification cycles of 95°C for 15 sec for denaturation, 58°C for 30 sec for annealing and 72°C for 30 sec for extension step.
  - The reference gene (housekeeping gene) was U6 RNA to calculate the relative expression levels of MicroRNA-215 and micro RNA- 141.
  - Primer sequence as shown in above Table 2.
  - The cycle threshold (CT) which is the number of cycles required for the fluorescent signal to cross the threshold in real-time PCR serving as a tool for calculation of the starting template amount in each sample. Gene fold expression changes are calculated using the equation $2^{-\Delta\Delta ct}$ using healthy controls as calibrator, where $\Delta\Delta Ct$ = [Ct (target, test)-Ct (reference, test)]-[Ct (target, calibrator)-Ct (reference, calibrator)] [25, 26,27].

2.5 Statistical Analysis

Obtained data were presented as mean±SD, ranges, numbers and ratios. Results were analyzed using One-way ANOVA with post-hoc Tukey HSD Test and Chi-square test (X^2 test). Possible relationships were investigated using Pearson linear regression. Sensitivity and positive predictive value (PPV=1-specificity) of estimated parameters as predictors were evaluated using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) compared versus the null hypothesis that AUC=0.05. Statistical analysis was conducted using the IBM SPSS Statistics, Version 23, 2015 (IBM Corp. Armonk, NY, USA) for Windows statistical package. P value <0.05 was considered statistically significant.

3. RESULTS

The study included 75 patients with hepatic diseases; 25 patients had liver cirrhosis

(Cirrhosis group), 25 patients with chronic hepatitis C (CHC group) and 25 patients with hepatocellular carcinoma (HCC group). The study also included 15 volunteers as control group. Patients of HCC group were significantly older and showed significantly: compromised liver function tests compared to controls and patients of other groups. Details of patients' enrolment data are shown in below Table 3.

All patients showed significantly higher serum AFP levels compared to controls. However, serum AFP levels were significantly higher in HCC patients than cirrhosis patient and CHC patients with significantly higher levels estimated in CHC patients compared to cirrhosis patients (Fig. 1).

Estimated relative expression levels of miR-215 were significantly higher in CHC and HCC patients compared to both controls and cirrhosis patients with non-significantly higher serum levels estimated in HCC patients compared to CHC patients and in cirrhotic patients compared to controls (Fig. 2).

On contrary, estimated relative expression levels of miR-141 were significantly lower in HCC and CHC patients compared to controls, while were non-significantly lower in cirrhotic compared to control. Mean serum miR-141 levels were significantly lower in HCC patients compared to cirrhotic patients, but were non-significantly lower compared to CHC patients with non-significant difference between cirrhotic and CHC patients (Table 4, Fig. 3).

Estimation of HCV RNA viral load in CHC patients showed that 7 patients had $\log_{10}$ H titer of ≤6 and 18 patients had $\log_{10}$ H titer was >6. Serum viral load showed positive significant correlation (r=0.475, p=0.016) with serum expression level of miR-215 (Fig. 4), while showed positive non-significant correlation (r=0.24, p=0.318) with miR-141 in CHC patients (Fig. 5).

Evaluation of the ability of estimated serum levels of MiR-215 and MiR-141 for differentiation between hepatic disease patients and controls, both markers showed that ability with AUC= 0.872 and 0.250, respectively (Fig. 6). In comparison to the AUC for the null hypothesis (the reference AUC=0.5), AUC for MiR-215 more significantly higher (p=0.0006), but AUC for MiR-141 was significantly lower (p=0.002).

On the other hand, estimated serum levels of MiR-215 showed high ability to differentiate between patients with liver cirrhosis from CHC patients with AUC= 0.899 that was significant versus the reference AUC, while estimated serum expression levels of MiR-141 showed AUC=0.438 which is non-significantly lower than the reference AUC (Fig. 7).

Evaluation of the ability of estimated serum levels of MiR-215 and MiR-141 for differentiation between HCC patients and patients with other hepatic disease showed that both markers are could identify HCC patients out of hepatic disease patients with AUC of 0.818 and 0.351, respectively (Fig. 8). In comparison to the reference AUC, AUC for MiR-215 more significantly higher (p=0.0007), but AUC for MiR-141 was significantly (p=0.009) lower than the AUC for the null hypothesis (Table 5).

Table 3. Demographic data and results of routine laboratory investigations of studied patients compared to controls

Group variable		Control	Cirrhosis	CHC	HCC
Age (years)		48.9±7.6	51.2±9.1	52.2±11.6	59.6±10.4*†‡
Gender; M:F		11:4	17:8	15:10	18:7
Liver function tests	AST (IU/ml)	32.05±6.4	42.1±7.4	60.4±18.7*†	110±13.9*†‡
	ALT (IU/ml)	28.3±3.9	50.8±7.9*	69.8±10.5*†	127.1±45.9*†‡
	TB (mg/dl)	1.07±0.08	1.5±0.7*	1.73±0.4*	1.89±0.4*†
	Albumin (g/dl)	4.5±0.4	3.93±0.35*	4.02±0.33*	4±0.34*
	INR	1.02±0.13	1.13±0.15	1.3±0.29*†	1.38±0.37*†
	AFP (ng/ml)	1.96±0.6	24.1±14.5*	173±72.8*†	461.7±49.3*†‡

*Data are presented as mean±SD; CHC: Chronic hepatitis C; HCC: Hepatocellular carcinoma; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TB: Total bilirubin; AFP: α-fetoprotein; * indicates significance versus control levels; † indicates significance versus levels estimated in cirrhotic patients; ‡ indicates significance versus levels estimated in CHC patients; p<0.05 indicates significant difference; p>0.05 indicates non-significant difference*

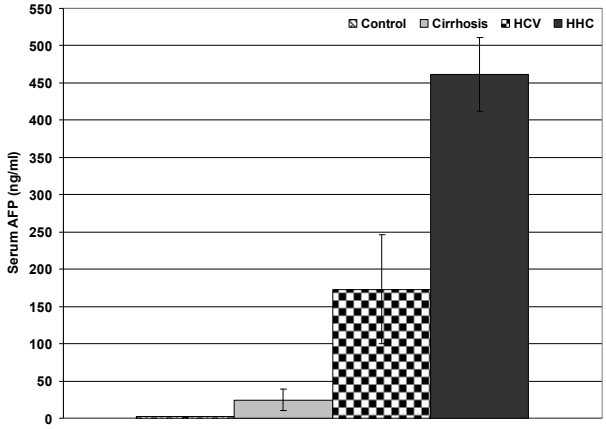

Fig. 1. Mean (+SD) serum AFP levels estimated in studied patients with varied liver diseases compared to controls

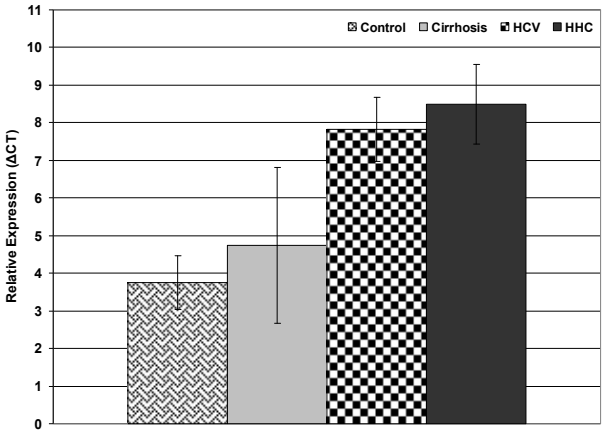

Fig. 2. The relative expression level of the circulating microRNA-215 in studied patients with varied liver diseases compared to controls

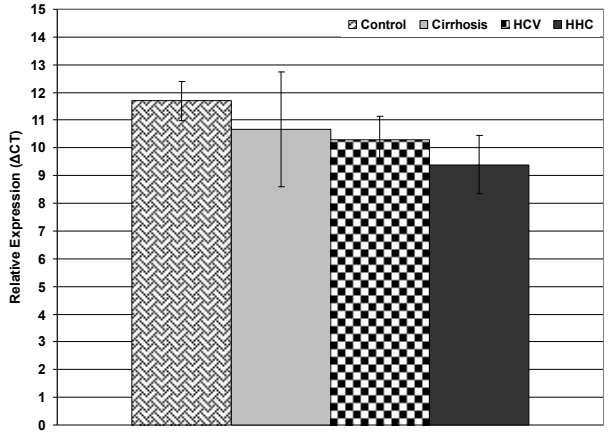

Fig. 3. The relative expression level of the circulating microRNA-141 in studied patients with varied liver diseases compared to controls

Table 4. Mean serum AFP level and relative expression levels of MiR-215 and MiR-141 estimated in studied groups

Parameter		Control	Cirrhosis	CHC	HCC
AFP (ng/ml)	Level	1.96±0.6	24.4±14.53	173±72.8	461.7±49.3
	P1		0.001	0.001	0.0001
	P2			=0.001	=0.001
	P3				=0.001
Relative expression level of MiR-215 (ΔCT)	Level	3.752±0.72	4.739±2.07	7.83±1.06	8.49±1.06
	P1		=0.072	=0.001	=0.001
	P2			=0.001	=0.001
	P3				=0.272
Relative expression level of MiR-141 (ΔCT)		11.7±1.56	10.68±1.53	10.28±1.85	9.39±1.45
	P1		=0.223	=0.042	=0.001
	P2			=0.799	=0.028
	P3				=0.212

Data are presented as mean±SD; AFP: α-fetoprotein; MiR: micro-RNA; CHC: Chronic hepatitis C; HCC: Hepatocellular carcinoma; P1 indicates significance versus control levels; P2 indicates significance versus levels estimated in cirrhotic patients; P3 indicates significance versus levels estimated in CHC patients; p<0.05 indicates significant difference; p>0.05 indicates non-significant difference

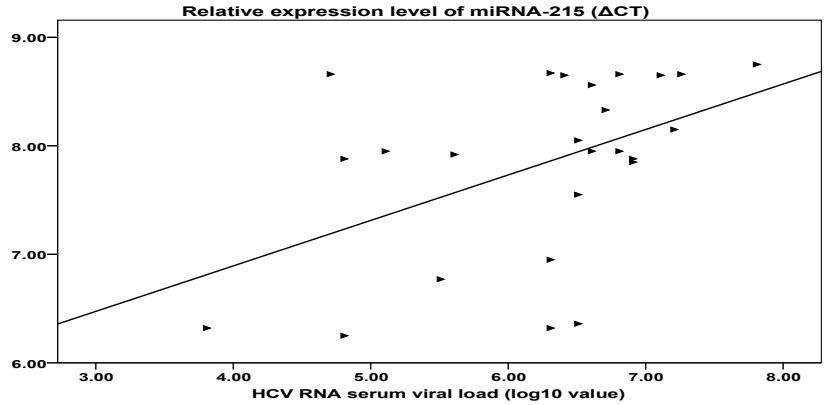

Fig. 4. Correlation between serum expression level of miR-215 and plasma HCV RNA viral load in patients with HCV

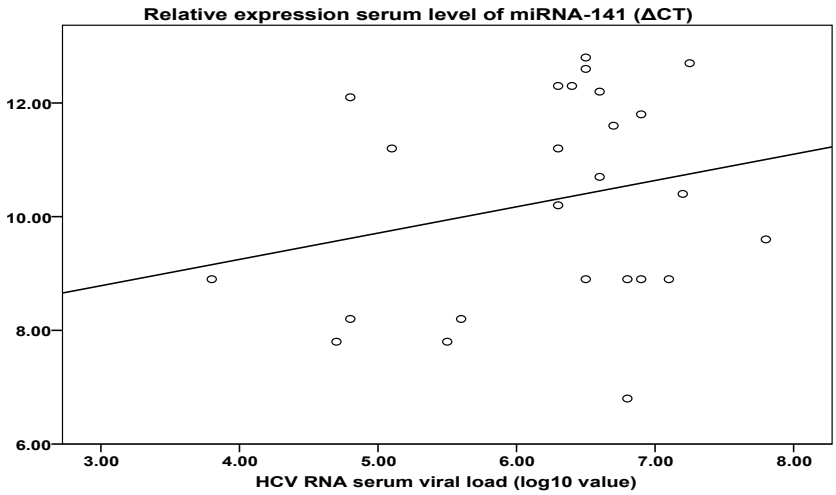

Fig. 5. Correlation between serum expression level of miR-141 and plasma HCV RNA viral load in patients with HCV

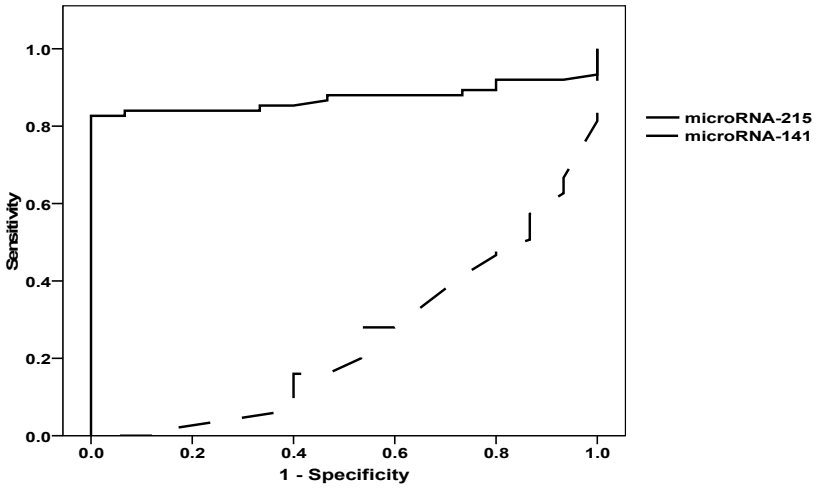

Fig. 6. ROC curve analysis of ability of estimation of serum expression level of MiR-215 and MiR-141 for differentiation between normal controls and patients had liver disease

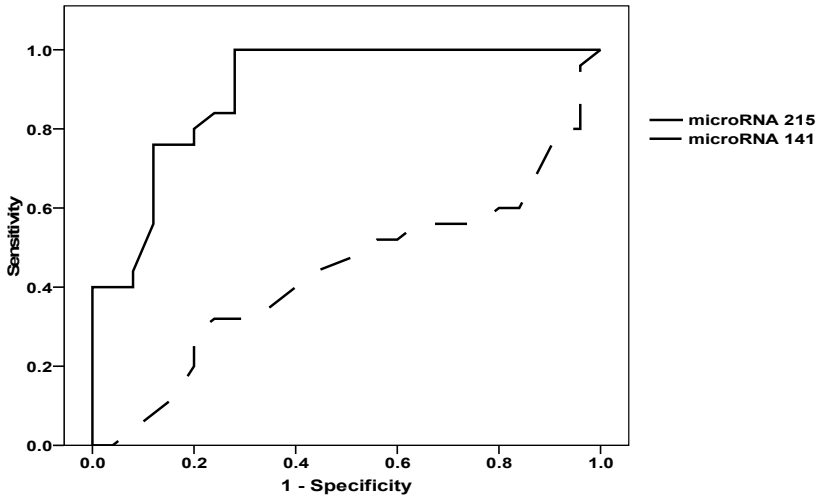

Fig. 7. ROC curve analysis of ability of estimation of serum expression level of MiR-215 and MiR-141 for differentiation between cirrhotic and CHC patients

Table 5. ROC curve analysis for applicability of estimation of serum expression levels of MiR-215 and MiR-141 for differentiation between patients with varied hepatic diseases

Purpose	Parameter	AUC	SE	P value	95% CI
Detection of hepatic disease	MiR-215	0.872	0.037	0.0006	0.800-0.944
	MiR-141	0.250	0.066	=0.002	0.120-0.380
Differentiation between CHC & cirrhosis	MiR-215	0.899	0.044	<0.001	0.814-0.985
	MiR-141	0.438	0.083	NS	0.275-0.600
Detection of HCC among liver disease patients	MiR-215	0.818	0.049	0.0007	0.722-0.915
	MiR-141	0.315	0.062	0.009	0.194-0.436

AUC: Area under curve; SE: Standard error; CI: Confidence interval; MiR: Micro-RNA: AUC>0.5 indicates high positive predictive value

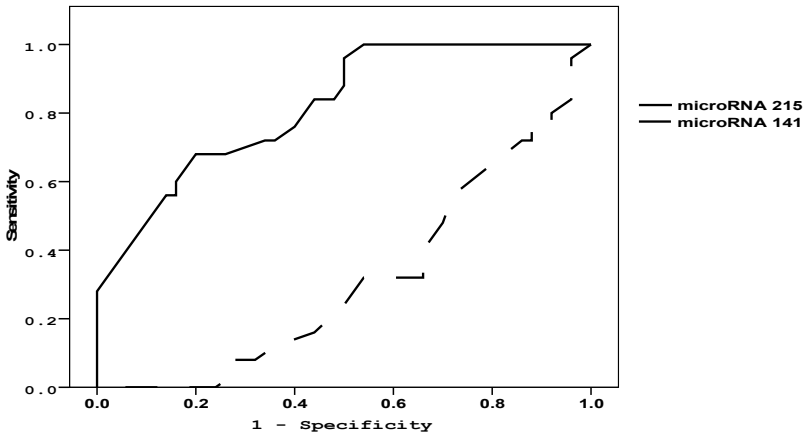

Fig. 8. ROC curve analysis of ability of estimation of serum expression level of MiR-215 and MiR-141 for differentiation between HCC and other hepatic diseases

4. DISCUSSION

Noninvasive tools for the diagnosis of cancer has became a goal of many researchers. Liver diseases, especially cirrhosis and CHC which are the precursors of HCC are associated with serum expression of varied miR [17,18,19]. Considering that miR-141 functions as a tumor suppressor and inhibits the migration and invasion of HCC cells [20] and miR-215 was deregulated with microvascular invasion or growth advantage in HCC patients [28], so the current study targeted to evaluate the ability of estimation of serum expression levels of miR-141 and miR-215 to differentiate between liver cirrhosis, chronic hepatitis C (CHC) and hepatocellular carcinoma (HCC) patients.

The obtained results fulfilled the aim of the study that estimation of serum expression levels of miR-141 and miR-215 can be used to differentiate between patients with liver diseases and free subjects on one side and HCC patients from patients with cirrhosis or CHC on the other side. These findings indicated the applicability of both miRs as serum markers for liver diseases, as non-invasive diagnostic modality for identification of HCC and supported that previous studies [12,29,30,31] concerning the use of miRs as markers of liver diseases especially HCC.

Estimated relative expression levels of miR-141 were significantly lower in HCC patients compared to controls and cirrhotic patients and in CHC patients compared to controls, while in cirrhotic patients estimated levels were non-significantly lower compared to the controls and non-significantly higher compared to CHC patients. These findings go in hand with the results of Liu et al. [32] findings using in situ hybridization analysis showing down-regulation of miR-141 in HCC tissues and cell lines and with Dhayat et al. [33] show circulating miR-200 family members are significantly deregulated in patients with HCC and liver cirrhosis. These data indicate a tumor suppressive role for miR-141 in HCC patients and so its decreased expression levels indicate progressive or increased severity of HCC. In support of this assumption, Lou et al. [34] found MiR-141 over expression resulted in significantly reduced cell proliferation, invasion, and migration effects on HCC cells.

In trial to explain the mechanism of tumor suppressive role for miR-141, Liu et al. [32] reported that miR-141 inhibits liver cancer cells by negatively regulating the T lymphoma invasion and metastasis 1 (Tiam1) gene, while Lin et al. [35] demonstrated that the repression of hepatocyte nuclear factor-3β by miR-141 suppressed the proliferation and invasion and promoted the apoptosis of HepG2 cells.

MiR-215, its estimated relative expression levels were significantly higher in CHC and HCC patients than controls and cirrhosis patients with non-significantly higher levels in HCC patients compared to CHC patients and in cirrhotic patients compared to controls. In line with these results Zhang et al. [36] detected significantly up-regulated expression levels of miR-143 and miR-215 in serum of patients with CHC and HCC. The currently obtained results spot light on a possible role of miR-215 in HCC tumorigensis and so its over-expression and high levels indicate increased disease severity. In line with this assumption, the current study detected positive significant correlation between serum viral load

and expression level of miR-215. To elucidate the mechanisms for miR-215 tumorigensis, Liu et al. [37] documented that HBV X (HBx) protein plays a vital role in the development of HCC through up-regulating miR-215. Wang et al. [38] demonstrated that the upregulation of miR-215 leads to the development of insensitivity to adriamycin and worsens the prognosis of HCC patients.

The ROC curve analysis of the obtained results assured the ability of estimated serum levels of miR-141 and miR-215 for differentiation between hepatic disease patients and controls and between HCC patients and patients with cirrhosis or CHC, while miR-215 only could differentiate between patients with liver cirrhosis from CHC patients. Similarly, Zhang et al. [36] demonstrated that ROC analyses detected the potential application of miR-143 and miR-215 in the diagnosis of HCC.

Moreover, serum expression levels of miRNA-215 were correlated with CHC serum viral load and so could be used as a diagnostic and prognostic marker for CHC patients and may replace frequent estimation of hepatitis viral C load. This finding goes in hand with Kumar et al. [39] detected significant positive correlation between micoRNA-122 level with and viral load.

The obtained data point to the increased diagnostic yield of estimation of serum levels of miR-141 and miR-215 for differentiation between liver diseases and identification of HCC patients. Serum AFP also showed significant higher levels in all patient groups compared to controls with significantly higher level in HCC patients than patients of cirrhosis and CHC groups. In support of this assumption multiple previous studies recommended the use of more than one marker for prediction, screening or diagnosis of HCC [40,41,42,43]. Recently, in 2017, Zhang et al. [16] documented that efficacy of the combination of 3-miRNA panel and AFP was powerful for HCC diagnosis, especially in early tumor screening.

5. CONCLUSION

Serum expression levels of MiR-215 and MiR-141 could identify hepatic disease patients with high PPV especially for MiR-215. MiR-215 can differentiate between cirrhosis and CHC patients and correlated with HCV load. Serum levels of both MiRs could assure diagnosis of HCC with high PPV. However, wider scale studies are mandatory for assurance of obtained results and identification of cutoff points for both MiRs to be applied as non-invasive diagnostic modalities.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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