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Circulatory miRNA-484, 524, 615 and 628 expression profiling in HCV mediated HCC among Egyptian patients; implications for diagnosis and staging of hepatic cirrhosis and fibrosis

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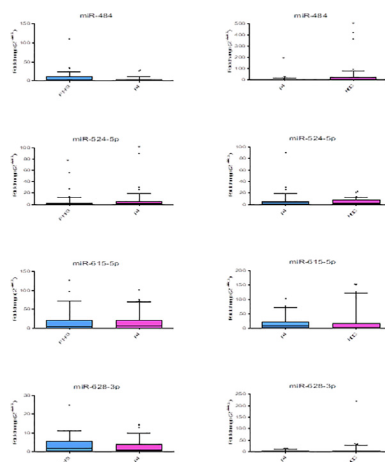
HIGHLIGHTS

- miR-484 is downregulated in advanced fibrosis as compared to mild fibrosis and HCC.
- miR-484 is upregulated in HCC as compared to cirrhosis.
- miR-524-5p is upregulated in cirrhosis and HCC.
- miR-615-5p is upregulated in cirrhotic group as compared to controls.
- miR-524 has promising discriminating power between cirrhosis and fibrosis.
- Studied miRNAs could be used in staging and diagnosis of hepatic HCV progression.

GRAPHICAL ABSTRACT



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ABSTRACT

Circulatory microRNAs have recently emerged as non-invasive and effective biomarkers for diagnosis of various diseases. Currently there is no reliable biomarker for diagnosis, prognosis or even staging of fibrotic and cirrhotic complications arising from HCV infection. This study aimed at investigating plasma miR-484, miR-524, miR-615-5p and miR-628-3p expression signatures in Egyptian patients with HCV mediated cirrhosis, fibrosis and HCC. Plasma miRNAs expressions in 168 samples [(40 healthy controls, 47 with HCV liver fibrosis, 40 with HCV-cirrhosis and 41 with HCV-hepatocellular carcinoma (HCC)] were quantified using RT-PCR. The studied miRNAs were differentially expressed among all participating groups. Plasma miR-484 levels exhibited significant downregulation in advanced fibrosis as compared to mild fibrosis and HCC. Moreover, miR-484 showed significant upregulation in HCC versus cirrhosis. Both miR-524-5p and miR-615-5p were upregulated in cirrhotic group as compared to controls.

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Cirrhosis
Biomarker

Differential expression between HCC and controls was noticeable in miR-524-5p. Receiver operator characteristic curve analysis revealed promising diagnostic performance for miR-484 in discriminating late fibrosis from both mild fibrosis and HCC and also for miR-524 in distinguishing between cirrhosis and fibrosis. In conclusion, investigated miRNAs could serve as potential and sensitive biomarkers for staging, prognosis and early diagnosis of various HCV mediated hepatic disease progression.

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Introduction

Hepatitis C virus (HCV) is a contagious liver disease affecting nearly 160 million people worldwide [1] In 2010, it was estimated that 0.5 million people die from HCV-associated hepatic diseases each year [2]. Nowadays and despite of the discovery of novel anti-HCV strategies, HCV associated death cases per year has doubled with more than 300 million people infected chronically with either hepatitis C or B [3]. Blood transfusion, hemodialysis, multiple drug injections and the use of unsterilized equipment are the main causes for increased prevalence of HCV especially in developing countries [4]. The global geographic distribution for HCV genotypes varies substantially in different parts of the world [5]. Egypt has high prevalence of HCV where approximately 3.5 million Egyptians have active HCV infection [6]. Genotype 4 is the most frequent form in HCV infected Egyptian patients [6]. HCV is a slowly progressing disease with persistent hepatic inflammation which could develop, in 20–30 years of HCV infection, into fibrotic wound scars, cirrhosis and/or ultimately hepatocellular carcinoma (HCC). The latter is the fifth most frequent cancer type globally and is ranked third in malignancy-related mortality cases [7]. It also represents 13% of common cancer types in Egypt with nearly 7000 death cases per year [8,9]. Various epidemiological studies showed that many HCC patients (as high as 70%) have anti-HCV antibody in the serum [10]. Currently available HCC diagnostic markers such as des-γ carboxy prothrombin, alpha fetoprotein (AFP), imaging and liver biopsy are either unreliable, insensitive, expensive or invasive [11,12]. Lack of cost effective, reproducible and non-invasive biomarkers for all HCV associated hepatic diseases is a major cause for late diagnosis and hence delayed intervention and/or therapy. Therefore there is an urgent need to search for alternative and reliable options that could assist in early diagnosis, prognosis and/or staging, with minimal invasion to suit late disease stages, prior to offering any therapeutic option.

MicroRNAs are small non-coding RNAs which regulate expression of genes in a sequence specific approach. They pair with mRNA targets at 3′ untranslated region (UTR) resulting in translational repression, mRNA de-adenylation and/or decay [13]. It is reported that miRNA control most of coding genes [14]. miRNA expression was found to be partly tissue specific [15]. For instance, miR-122 is mainly expressed in liver [16]. In HCV infection, HCV RNA sequesters hepatic miR-122 for its own replication which in turn depresses normal miR-122 targets [15]. Miravirsen, a locked nucleic acid, is an antagomir against miR-122 decreasing HCV replication and was therefore used successfully as the first miRNA dependent drug [17]. Circulatory miRNAs, in particular, have emerged as potential noninvasive biomarkers in various HCV mediated liver diseases. Elevated levels of miR-155 and miR-122 and decreased levels of miR-16 and miR-199a were correlated with pathogenesis of HCV mediated HCC and were considered to be reliable non-invasive HCC biomarkers [18]. However, limited studies are available for correlation of miRNA expression profiles with hepatic disease progression from HCV infection to fibrosis and cirrhosis. This study aimed at investigating expression profiles for circulatory miR484, miR-524-5p, miR-628-3p and miR-615-5p in the plasma of HCV mediated fibrosis, cirrhosis and HCC Egyptian patients as potential and non-invasive biomarkers for staging and early diagnosis purposes especially for fibrotic and cirrhotic cases.

Rationale of miRNAs selection:

Bimpaki et al. (2010) was the first to identify miR-484 as one of the upregulated miR in massive macronodular adrenocortical disease [19]. Vecchione et al. (2013) reported its down regulation in chemoresistant breast cancer [20]. Yang et al. (2016) found that miR-484 induced hepatocellular malignancy transformation in animal model and also cell lines [21]. Recently and on the contrary, Tessitore et al. (2016) reported that miR-484 was dysregulated in HCC liver tissues. This controversy could be attributed to use of different sample sources as miRNAs are partly tissue specific [22].

Bioinformatic analysis of in glioma cells revealed association of miR-524-5p with Hes-1 and Jagged-1 components of notch pathway. Using in vitro assay, miR-524-5p overexpression was reported to induce apoptosis and suppress cancer invasion and proliferation in glioma. It was therefore considered as a cancer suppressor by targeting different components in notch pathway [23]. It was also reported that its overexpression suppresses tumor proliferation through inhibition of the mitogen-activated protein kinase)MAPK(pathway [24].

Expression of miR-615-5p was reported to be upregulated in HCC and cirrhotic hepatic samples. Its expression was inversely correlated with Insulin-like growth factor 2 (IGF-II) mRNA expression through direct binding to the 3′-UTR region of IGF-II [25]. Its expression was elevated also in both HCC tissues and cell lines with negative regulation of serine hydroxymethyltransferase 2 (SHMT2) and its downstream targets [26]. Woo et al. (2016) reported SHMT2 upregulation in HCC cell lines that were knocked down for miR-615 and noticed reduction in tumorgenicity and cell proliferation [27]. On the other hand, Chen et al. (2017) reported downregulated for miR-615-5p in HCC tissues with increase of Ras-protein which enhanced HCC metastasis in-vivo and in-vitro models [28].

The miR-628-3p was first reported in enteroviral infection and was considered to moderately distinguish between infected and healthy individuals [29]. Later on, its expression patterns were found to be downregulated and upregulated in colorectal and pancreatic cancers respectively [30]. Yet, its association with cancer formation and progression in general and liver in particular is not illustrated.

The expression profiles for the four mentioned miRNAs were not previously investigated in the plasma of HCV mediated fibrosis, cirrhosis and HCC cases across the globe and among Egyptians in particular.

Patients and methods

Patients

Following implementation of rigorous inclusion and exclusion criteria, one hundred and twenty eight chronic HCV Egyptian patients were recruited in this study. All participants of this study

signed a written informed consent and the ethics review committee at the Faculty of Pharmacy, Cairo University approved the study protocol (Serial: BC1603). This study was carried out according to Helsinki ethical guidelines. The design of the study is observational cross-sectional. Patients with the following inclusion criteria were recruited in this study; age elder than 18 years old; positive evidence for HCV infection confirmed by both anti-HCV test (HCV Ab Plus Rapid Test, Cairo, Spectrum) and positive HCV viral titer as detected by Q-RTPCR (Step-One Plus, Applied Biosystems, California, USA). Patients were negative for both hepatitis B core Ab (HBc-Ab) and hepatitis B surface antigen (HBsAg). Exclusion criteria were applied for any patient younger than 18 years old, or any patient with positive HBsAg and/or HBc-Ab or any other co-infection with HBV, or any patient with chronic hepatitis or cirrhosis originating from any other disorder other than HCV infection, or any patient with secondary liver cancer or any other malignancy other than HCC, or any HCV patients who received any antiviral therapy, or any treated HCC patient. Fibrotic and cirrhotic patients were categorized based on their FibroScan scores which were represented in kilo pascal. HCC diagnosis was confirmed by multiphasic MRI and/or dynamic computed tomography. Patients were admitted, over the past three years, to the Endemic Medicine Department, Kasr El-Aini Hospital at the Faculty of Medicine, Cairo University. Based on the rigorous criteria set for inclusion and exclusion in this study, the 128 filtered patients were classified into 47 cases with liver fibrosis (F0 to F3) subcategorized as 26 mild fibrosis (F0-F1) and 21 advanced fibrosis (F2-F3), 40 cases with liver cirrhosis (F4) and 41 HCC cases. Forty healthy volunteers with negative anti-HCV test were recruited in this study as control group. All cases included in this study were subjected to clinical, serological and biochemical investigations.

Samples collection

Blood samples were collected and both serum and plasma were prepared for each sample. For plasma preparation, EDTA treated blood samples were firstly centrifuged at low speed at 4000 rpm for 10 min then the supernatant was centrifuged at high speed at 13,000 rpm for 10 min at 4 °C, to get rid of any cellular debris, and stored at −80 °C until use.

Routine laboratory tests

Routine lab tests included complete blood count (CBC), RBCs count, total leukocyte count, platelet count, using cytometer after dilution with appropriate solution for each assay (Egyptian Diagnostic Media, Egypt) hemoglobin (Hb) (Spectrum, Egypt), calculated MCV, prothrombin time (Biomed, Cairo, Egypt), international normalized ratio (INR), AST, ALT, ALP, bilirubin, albumin, alpha feto protein (AFP) using Spectrum® kit (Cairo, Egypt). All control samples were negative for HCV-antibody test.

RNA extraction, polyadenylation and cDNA synthesis

Direct-zol™ RNA MiniPrep Kit (Zymo Research, California, USA) was used to extract total RNA according to manufacturer's manual. During extraction, cel-miR-39-3p (Qiagen, Düsseldorf, Germany) was used as spike-in control for normalization purposes. The A260/A280 ratio was measured for extracted RNA to determine quality and quantity using the Q5000 UV–Vis Nanodrop (Quawell, California, USA). A total of 100 ng RNA was poly adenylated with *E. coli* Poly(A) Polymerase (New England Biolabs, Hitchin, UK) and then reverse transcribed to synthesize cDNA using GoScript Reverse Transcription kit (Promega, California, USA) as per manufacturer's protocol.

Quantitative reverse transcription-PCR

In each 20 μl PCR reaction of the 96 well plate, 2 μl of each diluted cDNA was mixed with 10 μl GoTaq® qPCR Master Mix (Promega, California, USA) and 0.4 μM of each primer. Primers have been carefully designed and validated for non-specific amplification to ensure precise quantitation for selected miRNAs. The plate was then amplified for 40 cycles and relative miRNA expression and fold change was calculated using $2^{-\Delta\Delta Ct}$ formula with the healthy individuals group as normalizer and the spiked miR-39 as internal control as follows; $^{\Delta\Delta}$Ct = [Ct (miR of concern, test)-Ct (miR-39, test)]-[Ct (miR of concern, calibrator)-Ct (miR-39, calibrator)].

Statistical analysis

Data was analyzed using SPSS software v-15 (SPSS, USA) and GraphPad Prism software V5 (GraphPad, California, USA). Data are represented as mean ± SEM. Analysis of variance (one way ANOVA), Tukey. Chi square and post hoc tests were utilized for comparison with P value ≤ 0.05 considered to be significant. Since miRNA fold change values were not normally distributed as computed by Shapiro-Wilk test, we performed Mann Whitney and Kruskal Wallis as non-parametric tests and data was presented

Table 1
Clinical and demographic features of all participating groups in this study: Data is represented as Mean ± SEM or number of cases (%) when appropriate. Data where analyzed using ANOVA or Chi-square test according to data type. Statistical significance is considered acceptable for *P*-value <0.05. Different letters indicate statistical significance between groups.

	Healthy (n = 40)	F0-F1 (n = 26)	F2-F3 (n = 21)	Cirrhosis (n = 40)	HCC (n = 41)	*P*-value
Gender						0.843
Male n (%)	24 (60)	15 (58)	14 (67)	21 (53)	24 (59)	
Female n (%)	16 (40)	11 (42)	7 (33)	19 (47)	17 (41)	
Age	47.25 ± 1.69^{A}	45.88 ± 2.23^{A}	47.76 ± 2.16^{A}	57.45 ± 0.92^{B}	60.65 ± 1.01^{B}	0.000
ALT	9.83 ± 1.28^{A}	34.26 ± 4.24^{AB}	35.19 ± 5.13^{AB}	48.49 ± 6.65^{BC}	69.67 ± 9.62^{C}	0.000
AST	13.74 ± 1.97^{A}	29.68 ± 3.06^{ABC}	38.63 ± 4.76^{ABC}	65.45 ± 7.85^{C}	110.37 ± 15.39^{D}	0.000
ALP	40.78 ± 2.81^{A}	73 ± 12.19^{AB}	113.33 ± 18.26^{AB}	169.22 ± 13.94^{B}	185.90 ± 17.70^{B}	0.000
AFP	Negative	4.19 ± 0.95^{A}	6.41 ± 1.88	10.08 ± 4.27	27.98 ± 8.13^{B}	0.012
Albumin	4.38 ± 0.11^{A}	4.07 ± 0.08^{A}	3.95 ± 0.12^{A}	2.68 ± 0.13^{B}	3.22 ± 0.11^{C}	0.000
Total Bilirubin	0.72 ± 0.09^{A}	0.72 ± 0.09^{AC}	0.89 ± 0.13^{AC}	4.06 ± 0.92^{B}	3.19 ± 0.68^{BC}	0.000
Prothrombin Time	15.93 ± 0.28^{A}	12.59 ± 0.22^{A}	13.88 ± 0.32^{A}	18.69 ± 0.79	17.46 ± 1.30^{A}	0.000
INR	1	1.12 ± 0.2	1.10 ± 0.03	1.59 ± 0.08	4.11 ± 2.59	0.580
Viral Load ($\times 10^5$)	Negative	11.73 ± 5.28	18.24 ± 6.84	14.68 ± 8.57	2.99 ± 0.92	0.457
Total Leukocytes Count ($\times 10/mm^3$)	5.14 ± 0.22^{A}	6.83 ± 0.38^{AB}	4.93 ± 0.37^{A}	7.04 ± 0.63^{B}	6.46 ± 0.42^{AB}	0.003
Platelet ($\times 10^3/mm^3$)	199.97 ± 9.10^{A}	213.8 ± 12.92^{A}	161.9 ± 16.89^{AB}	126.37 ± 12.64^{B}	129.04 ± 10.06^{B}	0.000
RBCs	5.26 ± 0.42	4.46 ± 0.26	5.07 ± 0.15	3.69 ± 0.13	4.09 ± 0.12	0.000
Hb	15 ± 1.15	13.51 ± 0.50	14.27 ± 0.36	10.79 ± 0.40	11.82 ± 0.32	0.000
MCV	87 ± 4.04	84.34 ± 3.08	80.41 ± 4.83	85.32 ± 1.75	83.07 ± 2.20	0.815

using median and quantiles. Analysis of correlation was carried out to investigate correlation between selected miRNAs in each category using Spearman correlation. Receiver operator characteristic (ROC) curves were plotted for evaluation of accuracy.

Results:

Clinical and demographic features of all participating groups in this study:

The clinical features for all studied individuals are shown in Table 1. In our analysis, chronic liver disease (CLD) group contained F0, F1, F2 and F3 patients. There was a significant trend ($P < 0.0001$) for progression of hepatic disease from fibrosis to cirrhosis and eventually to HCC with increasing age ($P < 0.0001$). No statistical difference for gender type among participating groups ($P = 0.843$) was observed. Serum biochemical parameters of ALT, ALP, AST, total bilirubin were significantly different among the studied groups ($P < 0.0001$) with increasing values towards disease progression till HCC. Albumin and prothrombin time tests showed significant decline in liver synthetic function with liver disease progression. Albumin was significantly lower in cirrhotic and HCC cases. Although INR values increased with disease progression, this increase was significant only (<0.0001) between HCC and the remaining groups. AFP showed significant difference between mild fibrosis and HCC ($P = 0.012$) but not between the other CLD groups. Hemoglobin, RBCs and platelet levels decreased significantly in all the studied groups as compared to the healthy controls ($P < 0.0001$). Major reductions in those levels were noticed in cirrhosis and HCC cases. On the other hand, total leukocyte count increased significantly in all patients. All healthy subjects had negative HCV viral titer while all the remaining groups had positive viral titer with slight non significant variation among them ($P = 0.457$). In principle, variation in HCV viral load could be attributed to several factors. One of those factors is the HCV

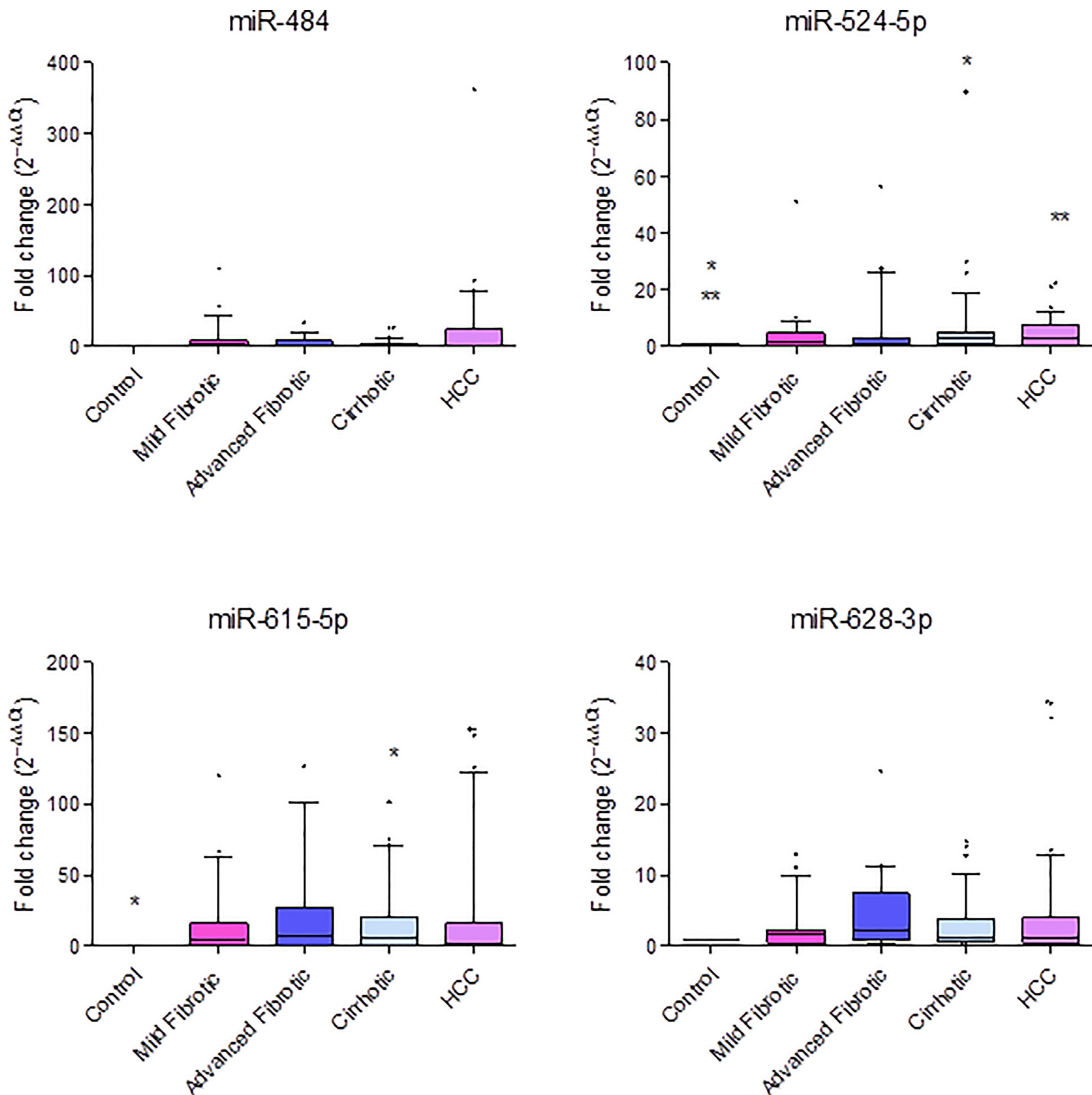

Fig. 1. Fold change and differential expression of plasma microRNA between different studied groups. * indicates significance difference (P < 0.05).

infection period, since viral load increases with time as concluded by Fanning et al. (2000) who noticed 1.7-fold increase in viral load per year in their studied patients [30]. Other factors that can cause variation in the baseline viral load are patients' age, BMI and their fibrotic stages as indicated by Ticehurst et al. (2007) who noticed significant correlation between baseline viral load and fibrotic stage, BMI and age among their studied patients [31]. Also, one of the factors that can influence the baseline viral load is gene polymorphism. Nguyen et al. (2018) reported significant association between interferon lambda gene polymorphisms and variation in HCV viral loads [32]. HCC patients recruited in this study had tumour size of <3 cm (20%), 3–5 cm (20%) and >5 cm (60%) as indicated by CT imaging. Forty eight percent of HCC patients had single liver focal lesion while the remaining had multiple hepatic foci. Occurrence of those lesions in the right lobe was recorded in 40% of HCC patients, 30% in the left lobe and 30% of cases had lesions in both lobes. Fifty eight of HCC patients developed portal vein thrombosis (PVT). The demographic and clinical data presented in Table 1 indicate that individuals recruited in each group are properly categorized and to perfectly represent the assigned group.

Differential expression of plasma miRNAs levels in all studied groups

The Kruskal Wallis test for multiple comparison of non-parametric data (GraphPad Software, CA) was used to examine if there is any statistical difference in the expression pattern of all

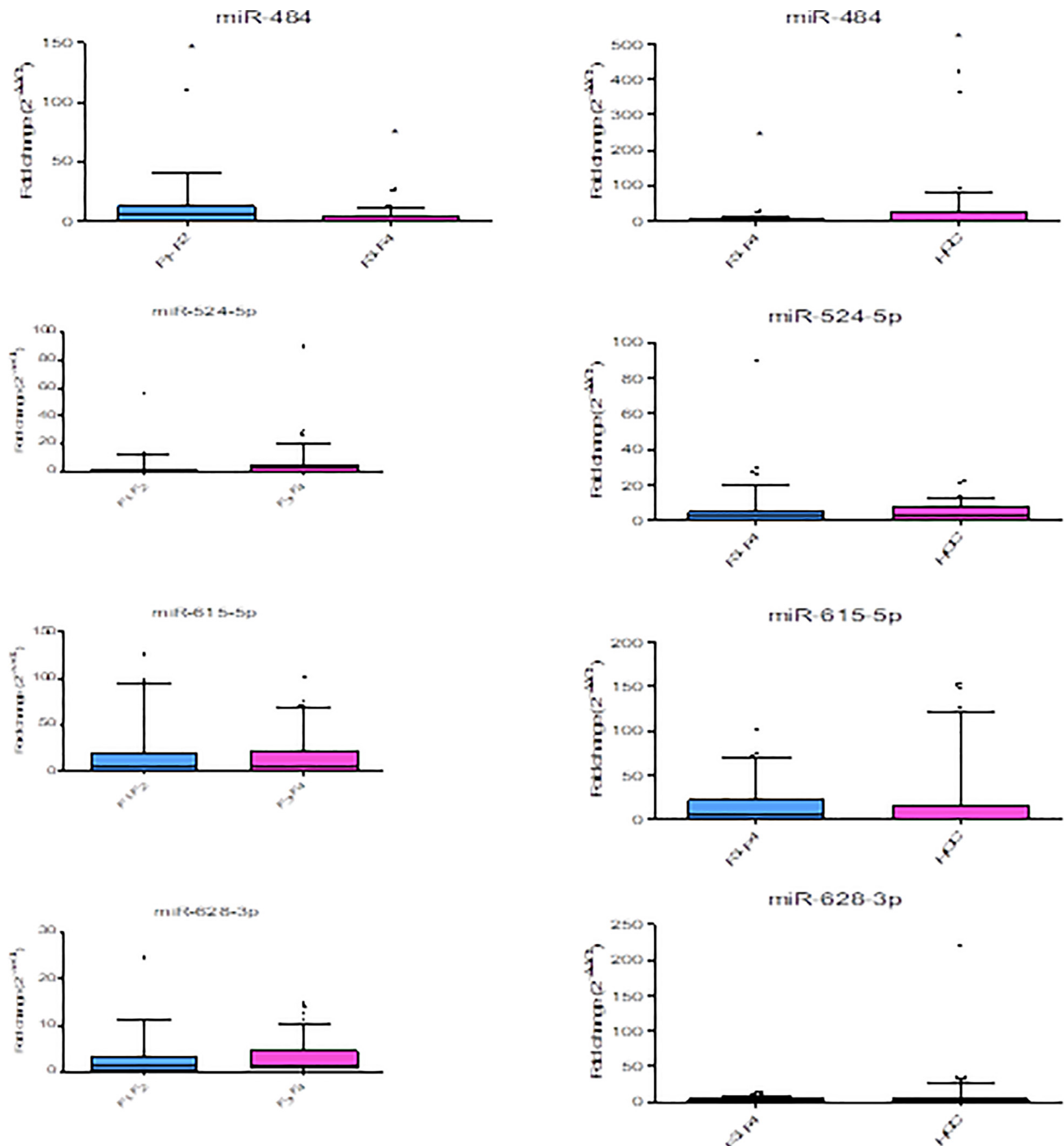

Fig. 2. Fold change and differential expression profiles for microRNA between mild fibrosis versus advanced fibrosis and between advanced fibrosis versus HCC. * indicates significance difference (P < 0.05).

the investigated miRNAs among healthy control, mild fibrosis, advanced fibrosis, cirrhosis and HCC. Plasma expression profiles for miR-615-5p and miR-524-5p in particular exhibited significant upregulation of 2.99 and 6.17 respectively between control group and patients with cirrhosis (P = 0.001 and 0.007 respectively) (Fig. 1). There was also significant increase (2.69, P = 0.001) in miR-524-5p expression pattern in HCC group as compared to healthy individuals. There was marginal significance among the studied groups for miR-628-3p (P = 0.067). No significant differential expression was observed for miR-484 among all the studied groups (P = 0.200).

Plasma miRNAs signatures across various stages of HCV mediated hepatic diseases

Furthermore, plasma miRNA expression profiles and signatures through progression from mild fibrosis (F1-F2) to advanced fibrosis (F3-F4) and eventually to hepatocellular carcinoma were investi-

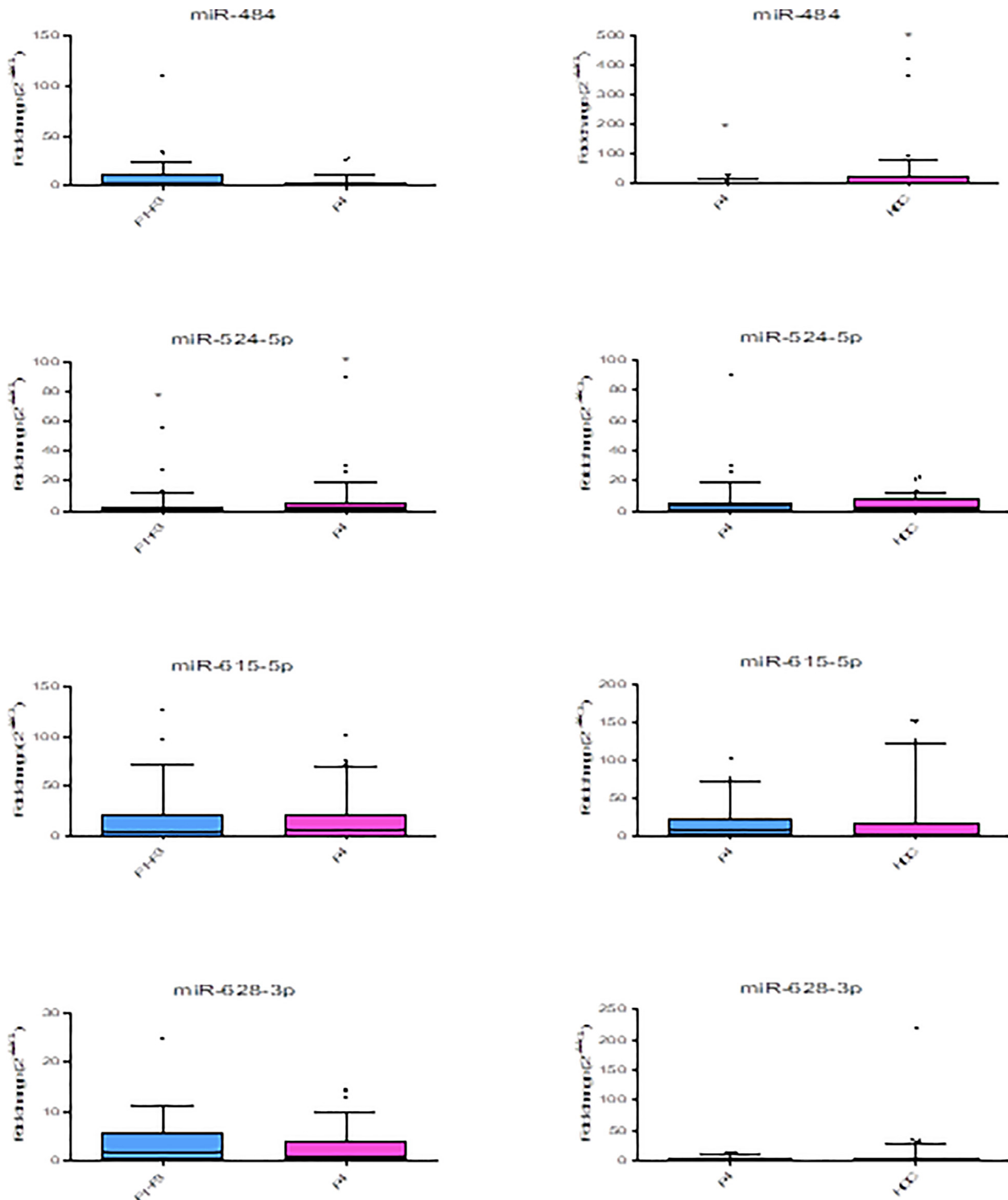

Fig. 3. Fold change and differential expression profiles for microRNA between (F1-F3) versus F4 and F4 versus HCC. * indicates significance difference ($P < 0.05$).

gated using Mann-Whitney *U* test. There was a significant reduction in miR-484 expression as F1-F2 patients progress to F3-F4 (from 6.23 to 0.27) ($P = 0.041$) (Fig. 2). Furthermore, HCC patients showed significant upregulation in miR-484 when compared to patients of F3-F4 category (from 0.27 to 1.25) ($P = 0.037$) as shown in (Fig. 2). The upregulation in HCC cases exceeded even that in F1-F2 cases. Other comparisons of F1-F2 versus F3-F4, showed non-significant upregulation for miR-524, miR-615-5p and miR-628-3p with *P*-values of 0.078, 0.780 and 0.608 respectively. As for comparison of F3-F4 versus HCC, no statistical significance was found for downregulation of miR-524-5p, miR-615-5p and miR-628-3p ($P = 0.798$, 0.083 and 0.519 respectively).

Moreover, individual plasma miRNA expression pattern in each stage of the fibrotic cases (from F1 to F4) was investigated using Kruskal-Wallis test (GraphPad Software, CA). There was no significant difference among those cases ($P > 0.05$). In further analysis, we also traced the profile signatures for the studied miRNAs in the combined fibrotic group (three stages from F1 to F3) against those of cirrhosis (F4 stage) and HCC cases through a dual comparison using Mann-Whitney *U* test (Fig. 3). miR-524-5p was fund to be significantly upregulated in F4 versus F1-F3 (2.99 and 0.91 respectively) ($P = 0.023$). Furthermore, miR-484 was upregulated in HCC versus F4 (1.25 and 0.27 respectively) ($P = 0.04$). The expression patterns for miR-615-5p and miR-628-3p did not exhibit significant difference ($P > 0.05$) neither for HCC versus F4 nor for F1-F3 versus F4.

Plasma miRNAs expression levels in hepatocellular carcinoma patients

None of the investigated miRNAs exhibited significant difference in expression profiles between HCC and CLD groups as confirmed by Mann-Whitney *U* test (Fig. 4). There was significant down-regulation for miR-615 expression in HCC group as compared to the CLD group (1.74 versus 6.17 median fold change respectively). Although the HCC group showed upregulation in their expression profiles of in miR-484, miR-524-5p and miR-628 as compared to the CLD group, but those upregulations were not significant ($P = 0.11$, 0.39 and 0.45 respectively).

Diagnostic performance of plasma miRNA

Receiver Operating Characteristic (ROC) analysis was conducted to examine the diagnostic performance of at all miRNA that showed significant differential expression (Fig. 5). ROC analysis for plasma miR-484 showed AUC value of 0.67 (95% CI 0.5067–0.8307, $P = 0.040$) between F3-F4 and F1-F2 categories which showed differential gene expression. The discriminating power for miR-524-5p to differentiate between F1-F3 versus F4 was investigated. ROC analysis showed an AUC = 0.66 (95% CI 0.5254–0.8050, $P = 0.022$). Furthermore, ROC curve analysis revealed also ability of the miR-484 to discriminate between HCC and F3-F4 with AUC value of 0.67 (95% CI 0.5067–0.8307, $P = 0.040$).

Logistic regression analysis of all the studies miRNAs

Univariate logistic regression (LR) for all the investigated miRNAs was carried out to predict miRNAs associated with hepatitis C virus-related hepatocellular diagnosis but with no significant values obtained. Furthermore, LR analysis for predictor miRNAs in cirrhotic cases revealed marginal significance for miR-484 only with a *P*-value of 0.083. On the other hand, LR analysis for chronic and fibrotic HCV patients exhibited non significant pattern with *P*-values of 0.059, 0.096, 0.682 and 0.857 for miR-484, miR-628, miR-615 and miR-524 respectively.

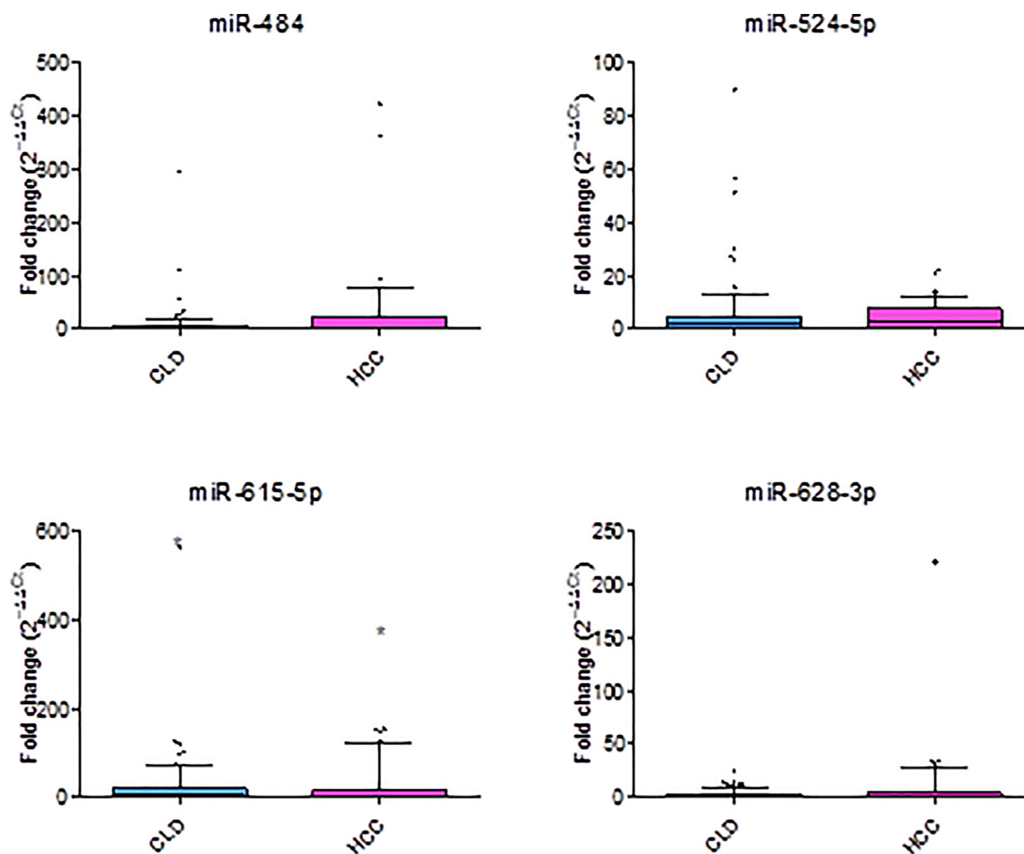

Fig. 4. Fold change and differential expression of plasma microRNA between HCC and CLD groups. * indicates significance difference ($P < 0.05$).

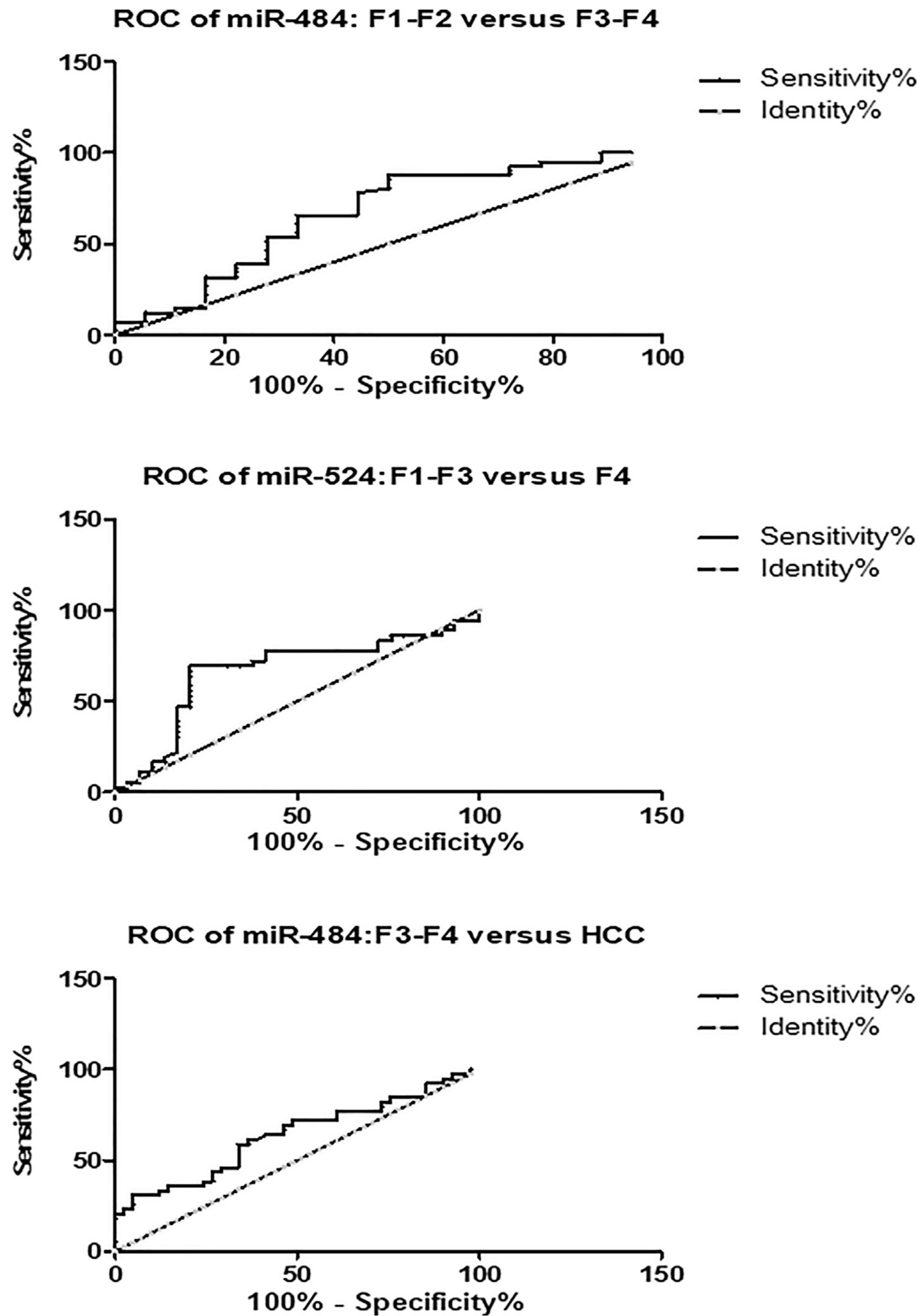

Fig. 5. Receiver Operating Characteristic (ROC) curve analysis for plasma miRNAs 484 and 524-5p.

Spearman correlations for all investigated plasma miRNAs

The investigated miRNAs exhibited positive and significant correlation among the fibrotic groups. miR-524-5p was correlated with miR-628-3p (Spearman r = 0.342, $P = 0.012$). There was no correlation between studied miRNA in either HCC or cirrhotic group with the highest correlation observed between miR-615-5p and miR-484 (Spearman r = 0.229, $P = 0.140$) and between miR-615-5p and miR-628-3p (Spearman r = 0.185, $P = 0.203$). Correlation study between HCV viral load and the investigated miRNAs revealed non significant association ($P > 0.05$) with correlation coefficient of 0.165, 0.182, 0.289 and 0.302 for miR-484, miR-524, miR-615 and miR-628 respectively.

Discussion

Prognosis and early diagnosis of fibrosis, cirrhosis and HCC disorders which are associated with prolonged HCV infection necessitates the urgent need to screen for reliable markers that can accurately detect those disorders and preferably their progression prior to offering proper medical intervention. Currently there is no non-invasive and reliable biomarker which could be utilized for diagnosis, prognosis or staging of fibrotic and cirrhotic complications arising from HCV infection.

In this study, the investigated miRNAs were differentially expressed among all participating groups. Plasma miR-484 levels showed upregulation in mild fibrosis (F1-F2) when compared to

advanced fibrosis (F3-F4) and also upregulation in HCC group when compared to advanced fibrosis (F3-F4). Plasma expression for miR-484 could discriminate between advanced fibrosis (F3-F4) and HCC cases. Furthermore, miR-484 showed significant increase in HCC versus cirrhosis (F4). The upregulation of miR-484 expression in HCC group comes in agreement with Yang et al. (2016) findings [21]. They performed an experiment using animal model and cell lines to examine the contribution of miR-484 to hepatocellular nodules formation but not with cirrhosis or fibrosis. miR-484 was found to induce hepatocellular malignant transformation. Our findings contradict those reported by Tessitore et al. (2016) who reported that miR-484 was dysregulated in HCC disease [22].

Levels of miR-524-5p were significantly upregulated in cirrhosis group versus healthy controls. It also showed upregulation in HCC as compared to cirrhosis with differential expression as compared to control. To the best of our knowledge, miR-524-5p expression was not reported previously neither in HCC nor in fibrosis nor in cirrhosis. In our study, miR-524-5p was downregulated in combined fibrosis (F1-F3) versus cirrhosis patients. Most of the other work published, showed downregulation of miR-524 in other cancer types such as glioma [23], but none evaluated its expression in HCC disease.

In this study, plasma miR-615-5p was upregulated in cirrhosis group when compared to control. Another study conducted by El Tayebi et al. (2012) reported that miR-615-5p was overexpressed in cirrhotic liver tissues as compared to healthy liver tissue [25]. It was proven that miR-615-5p suppresses IGF-2 which in turn reduce invasion and proliferation of HCC [25]. Therefore it was believed that miR-615-5p acts as tumor suppressor targeting IGF-2 win various tumor types [25]. In this study and for the first time to be reported, there was upregulation in mild fibrosis (F1-F2) group versus control group. It was also upregulated in F3-F4 when compared to healthy controls. Moreover significant downregulation for miR-615 expression in HCC group as compared to the CLD group was also reported for the first time.

Concerning miR-628-3p, there was marginal significance for differential expression among the Egyptian studied groups of HCC, cirrhosis and fibrosis as compared to the healthy individuals ($P = 0.067$). It was also positively correlated with miR-524-5p was correlated (Spearman $r = 0.342$, $P = 0.012$). Expression profile for miR-628-3p was reported to be upregulated and downregulated in pancreatic and colorectal cancers respectively [33].

TargetScan and miRDB programs were also used in this study to predict miRNA targets genes. The two programs revealed that miR-484 targets various key cell regulators such as protein tyrosine phosphatase receptor type E (PTPRE), transforming growth factor beta receptor 3 (TGFBR3), CREB regulated transcription coactivator 3 (CREB3L3) and membrane associated guanylate kinase, WW and PDZ domain containing 1 (MAGI1). PTPRE is a member of tyrosine phosphatases which regulates cellular growth and oncogenic transformation. CREB is cAMP dependent transcription factor which play a key role in hepatocellular carcinoma. TGFBR3 is a membrane proteoglycan which inhibits TGFB signalling and its downregulation is associated with cancer development. MAGI1-participates in cell–cell contact through assembly of several protein complexes on the plasma membrane. Concerning miR-524, it was found to bind mitogen-activated protein kinase 1 and 4 (MAPK1 and MAPK4), growth factor receptor bound protein 10 (GRB10) and BCL6 corepressor like 1 (BCORL1). MAPK family is also named as extracellular signal regulated kinase (ERK) which regulates various transcription factors and cellular proliferation. GRB10 suppresses cell growth through inhibition of tyrosine kinase signalling pathway. BCORL1 represses transcription through interaction with various classes of histone deacetylases. Targetscan and miRDB programs also identified ABI family member 3 (ABI3), Adenomatosis polyposis coli 2 (APC2), IGF-2 and ETS proto-oncogene 1, transcription factor (ETS1) as main target genes for miR-615. ABI3 inhibits cell migration and metastasis. APC2 reduces beta-catenin levels which plays a key role in cancer pathogenesis. ETS1 is a transcription factor which reulates various biological processes which control cell development and tumorigenesis. Amon the key target genes of miR-628 was BCL2 interacting protein 3 like (BNIP3L), EFR3 homolog 3 (EFR3A) and Serine/Threonine Kinase 40 (STK40). BNIP3L is a member of the pro-apoptotic subfamily. EFR3A controls activity of G protein coupled receptors. While STK40 regulates various signalling pathways associated with versatile activities of the cell such as survival, proliferation and apoptosis.

In this study, significant positive correlation was reported between the studied miRNAs among the fibrotic groups using RT-PCR. The four investigated miRNAs of this study could discriminate different stages of HCV associated diseases from healthy individuals and could therefore have promising diagnostic value. Moreover, ROC analysis revealed that those miRNAs could be used to distinguish advanced fibrosis from mild cases. This in turn suggests the possibility of utilizing those miRNAs as promising and sensitive biomarkers in HCV mediated liver disease progression. Conducting this experiment on larger population size and also on other populations from other ethnicities with different viral genotype infection would be beneficial prior to selecting those miRNAs as universal markers in HCV mediated liver disease progression. Screening further other miRNAs using more sophisticated and highthroughput techniques such as microarray and next generation sequencing (NGS) would definitely enrich the biomarker library and would facilitate the process for optimal selection of ideal miRNA panels for early diagnosis, staging and prognosis at universal level.

Conclusion

For the first time, our study profiled expression signature of miR-484, miR-524, miR-615 and miR-628 in HCV Egyptian patients at various disease stages. The investigated miRNAs were expressed differently in the plasma of HCC cases and could discriminate severe fibrotic cases from mild fibrosis and cirrhosis. Plasma miR-484 showed a unique expression profile with upregulation in mild fibrotic cases followed by downregulation as cases develop advanced fibrosis and is then upregulated in HCC cases. Expression level for miR-524-5p was upregulated in both cirrhosis and HCC cases following downregulation in F1-F3 combined fibrosis. Plasma miR-615-5p was upregulated in cirrhosis group when compared to control. Therefore those studied miRNAs can be beneficial in staging and diagnosis of various fibrotic, cirrhotic and HCC complications arising from HCV infection. The newly studied miRNAs could be utilized in further clinical evaluation in other populations of different genotypes.

Compliance with ethics requirements

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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Author contribution

AAG and SAM conceived and designed the experiments. SME, NZ and AY provided samples, recruited patients and provided some of patients' clinical data. OA and AAG performed experimental work and statistical analysis. AAG designed primers of this study. AAG and OA drafted the manuscript. SAM, OA, NZ, AY, SME and AAG revised the manuscript.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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