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SERUM MICRORNA-122 LEVELS IN EGYPTIAN PATIENTS WITH CHRONIC HEPATITIS C VIRUS GENOTYPE 4 INFECTION BEFORE AND AFTER TREATMENT WITH DIRECT ACTING ANTIVIRAL DRUGS

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Abstract

Background: The seventh leading cause of mortality worldwide was assessed as viral hepatitis. Approximately, 50% of mortality attribute to HCV, primary led to liver cirrhosis and tumor. Recently developing of high effective oral DAAs provided opportunity to decreased HCV diseases load and their onward transmission. With the possibility of eradicating HCV, the 'Global Health Sector Strategy on Viral Hepatitis 2016-2021 has recently been formulated by the WHO to eradicate HCV as a public health concern by 2030.MicroRNAs are small noncoding regulatory RNAs and circulate in blood in cell-free stable forms. MiR-122 levels commonly decreased in hepatocellular carcinoma comparing to normal liver and low miR-122 levels associated by bad prognosis. Overexpression of miR-122 diminishes tumorigenic properties in HCC cell lines, signifying its function as tumor suppressor gene.

Objective: to evaluate miR-122 level at end of HCV treatment by DAAs and compare the results with miR-122 level in HCC patients, to assess the risk of HCC after DAAs treatment.

Patients and Methods: A prospective case control study held at Ain Shams University hospital. Forty participants were included and further divided into:

Group A: (10) HCV -ve participants as control group,

Group B: (10) HCV infected patients with HCC,

Group C: (10) HCV infected patients (F1, F2) treated with sofosbuvir, daclatasvir +/-Ribavirin for 12 weeks

Group D: (10) HCV infected patients (F3, F4) treated with sofosbuvir, daclatasvir +/-Ribavirin for 12 weeks.

Results: Baseline MiR-122 level was significantly lower in HCC patients than chronic HCV patients and normal controls with cutoff value ≤0.26 showing 80% sensitivity and 70% specificity. MiR-122 was significantly reduced at end of HCV therapy with DAAs in (F3, F4) patients than those with early fibrosis stages (F1, F2).

Conclusion: HCV treatment with DAAs leads to miRNA 122 reduction more in (F3, F4) patients than those with early fibrosis stages (F1, F2), which may be related to hepatocarcinogenesis.

Keywords: Hepatitis C virus, chronic hepatitis, Serum MicroRNAs, Direct acting antiviral drugs, Hepatocellular carcinoma

INTRODUCTION

Hepatitis C virus (HCV) infections are the most reason for most liver diseases. It is a substantial health problem worldwide. According to WHO reports, 73 million people globally were infected by HCV (1).

HCV genotypes 1, 2 and 3 are spread globally, whereas genotypes 4, 5 and 6 are present in certain areas of the world. HCV patients mostly were remained chronically infected and increasing cirrhosis and hepatoma risks. New direct acting antiviral drugs (DAAs), design for inhibiting 3 viral proteins (NS3/4A protease, NS5A protein, and NS5B RNA dependent RNA polymerase) (2).

Egypt is one among the most affected countries by HCV. Egypt as developing country has national strategy to controlling of HCV and treatment program (3). Post successed negotiations for 99% discounted DAA prices, Egypt launched ambitious national HCV treatment for more than 250,000 chronically infected annually (4).

Hepatocellular carcinoma is major malignant tumors. Its new tumor cases increased every year and HCC almost runs fulminant course with grave prognosis. High relapse rates post surgical interference with poor response to anticancer drugs and radiotherapy (5).

MicroRNAs are small noncoding regulatory RNAs that circulated in blood in cell-free stable forms within small vesicles or protein complex (6). Circulating pattern extracellular miRNAs noted to altered in many diseases (7).

Extracellular levels of liver-derived miRNAs in particular miR-122, a miRNA that accounts for 50–70% of all miRNAs in the liver, (8) is emerged and highly specific parameters in drug-induced liver injury cases or chronic viral hepatitis B or C. MiR-122 known to regulated genes expressions as hepatic lipid and cholesterol metabolism (9) and HCV replication and apoptosis. miR-122 persistent expression appear as essentially to maintain adult liver phenotype and tumor suppressors (10). Also, miR-122 for replication of HCV prompt developing of antisense inhibitors of miR-122 (11) and MicroRNA dysregulation involved in hepatocarcinogenesis stages, and microRNA profiles had potential to differentiated HCC cases from healthy subjects (12). MiR-122 commonly low in HCC comparing with normal liver and which would associated with poor prognosis (10).

Previous studies suspected that HCV therapy by DAAs may increase risk of HCC (13,14). Many studies showed contradicting results as the effect of treatment of HCV by DAAs on HCC has been controversial (15,16) so in the present study we evaluate microRNA-122 level before and after HCV treatment by DAAs and compare the results with micoRNA -122 level in HCC patients. Our secondary aim was to determine the accuracy of miRNA-122 in predicting HCC.

PATIENTS AND METHODS

The study was performed in Ain Shams University hospital outpatient clinic in Egypt in the period between August to October 2019.

Forty participants in our study are divided to:

Group A: 10 HCV -ve participants as control group, ***Group B:*** 10 HCV infected patients with HCC, ***Group C:***10 HCV infected patients with early stage of liver fibrosis (F1, F2) treated with sofosbuvir (400 mg tablet once daily), daclatasvir (60 mg tablet once daily) plus ribavirin if indicated for 12 weeks and ***Group D:*** 10 HCV infected patients with late stage of liver fibrosis (F3, F4) treated with sofosbuvir (400 mg tablet once daily), daclatasvir (60 mg tablet once daily) plus ribavirin if indicated for 12 weeks.

Inclusion criteria to HCV groups (C, D):
Chronic naïve HCV infected patients eligible to treatment by DAAs.

Exclusion criteria to HCV groups (C, D):
Patients with age below 18 or above 70 years. Patients with decompensated liver cirrhosis, DM, Alcoholic liver disease and HBV.

All included subjects were subjected to thorough history, physical examination and serum anti-HCV antibody testing by ELISA (enzyme-linked immunosorbent assay (automative), test was done by Evolis Twin plus (BIO-RAD) equipment made in France 2004).

Full blood count, liver function tests (including albumin, bilirubin, prothrombin time, AST, ALT) before and every four weeks till end of treatment, serum creatinine and Alphafetoprotein (AFP).

Detection of the degree of fibrosis was done by FIB-4 calculation (Age x AST) / (Platelets x √ALT). Threshold value of < 1.45 had sensitivity of 74% and specificity of 80% in excluding fibrosis (F0-F1) and more than 3.25 had specificity of 98% in confirming cirrhosis (F3-F4) (17).

Pelvi-abdominal U/ S by TOSHIBA diagnostic ultrasound Xario200 made in Japan in 2014 by Toshiba Medical Systems Corporation. Abdominal triphasic C.T. with contrast was done for HCC patients. CT was done in different phases of contrast enhancement (18).
Quantitative PCR HCV RNA was done before treatment, at 4 weeks of treatment and at end of treatment. PCR is used in tube and when completed reaction where its products analyzed and visualized.
HCV RNA extracted from 220-µl aliquots. Extraction done per manufacturer's directions with some modifications1-, 15.5µl of HCV RNA ASR with initial concentration of 4.38×10^6 IU/ml add to lysis buffer contained carrier RNA and dilut in dilution buffer (80µl). Abbott internal QS add to lysis buffer for stoping degradation. HCV RNA ASR contain RNA sequences complementary to primers used to amplify HCV and intervening scrambled sequences which using for HCV RNA ASR detection. HCV RNA ASR used for HCV RNA quantifications (19).

Assay of Serum microRNA 122
This parameter was determined for all groups (A, B, C and D) baseline level and at the end of treatment to groups (C, D). RNA isolate from 200 µl of serum by used miRNeasy Serum/Plasma Kit (Germany). Isolations was performed accorded to manufacturer's instructions. Following phenol chloroform phase separation, interphase and organic layers rehydrated with RNAse free water equal to volume removed through aqueous phase collection. Aqueous phase achieved by maximizing RNA recovery. Both of them were

combined to 1 RNeasy MiniElute spin column and RNA isolation. cDNA diluted in 10-fold prior to microRNA PCR array run. through validation phase, serum RNA extract by used Plasma/Serum RNA Purification Mini Kit (Canada). Prior to transfer, sample onto Micro Spin Column the mixture pass throughout syringe by 26G needle to break down lysis debris and prevent column clogging. RNA eluted in RNAse (15µl) free water and assess on NanodropTM. RNA Extraction reverse transcribed by used miRCURY LNATM universal RT microRNA PCR Kit (Denmark). Fixed RNA volume input of 4 µL used to limitations in quantifying circulatory microRNAs. cDNA products dilute 1:40 prior to qRTPCR use (20). RQ expression levels calculated by used comparative method (Biosystems 7500 software version 2.0.1). Quantity of target miRNA-122 is normalized to endogenous reference U6 small RNA and relative to control.

All cases provided written informed consent to participating in our investigation, and our investigation approved by ethical committee of Ain Shams University

Statistical analysis
Data analyzed by using SPSS (version 16) (21). Student's t-test for indicated any significant differences among studied groups for normally distributed. Mann-Whitney test using for collectively indicate of any significant differences among studied groups for not normally distributed.
P<0.05.

RESULTS:

The study was conducted on 40 subjects divided into 4 groups within the age range of 25 to 70 years with male: female ratio of 3:2.

Laboratory parameters studied showed that haemoglobin level **(Hb)** was significantly lower in HCC group B (11.1±1.24 g/dl) than both (F1, F2) groups C (13.7±1.41 g/dl) and (F3, F4) D (13.32±0.98 g/dl) (p=0.003). Additionally, platelet count **(PLT)** was significantly lower in HCC group B than other groups A, C and D. **AST level** elevated in HCC group B comparing with healthy group A and higher in group D than groups A and C. **Serum Albumin** was significantly lower in HCC group B (2.76±0.336 g/dl) than other groups (p<0.001). **Serum Bilirubin** and **INR** showed significant increase in HCC group B than other groups.
Undoubtedly, Alphafetoprotein (**AFP)** is higher in HCC than other groups.

Table (1): Comparison between levels of microRNA-122 in the studied groups

microRNA 122		Groups				ANOVA	
		A	B	C	D	F	P
Baseline (Before therapy)	Range	1.23 - 2.53	0.103 - 0.743	2.95 - 5.53	1.75 - 3.72	37.528	<0.001*
	Mean ±SD	1.890 ± 0.514	0.294 ± 0.259	4.242 ± 0.903	2.846 ± 0.692		
After end of therapy	Range	-	-	0.372 - 2.345	0.023 - 0.932	12.619	<0.001*
	Mean ±SD	-	-	1.198 ± 0.704	0.377 ± 0.383		
Differences	Mean ±SD	-	-	3.045 ± 0.964	2.469 ± 0.523		
Paired Test	P-value	-	-	<0.001*	<0.001*		

TUKEY'S Test						
	A&B	A&C	A&D	B&C	B&D	C&D
Before TTT	0.007*	<0.001*	0.089	<0.001*	<0.001*	0.001*
After TTT	-	0.099	<0.001*	0.020*	0.991	0.008*

Baseline microRNA- 122 level was significantly lower in (HCC) group B than other groups (p <0.001). It was also significantly higher in (F1, F2) group C than healthy group A and (F3, F4) group D.

After end of HCV therapy

MicroRNA-122 showed significantly lower levels in patients with F3, F4 fibrosis (group D) than patients withF1, F2 hepatic fibrosis (group C) and controls (group A) with (P=0.008) and(P<0.001) respectively. differences were not significant in MiRNA-122 level in patients of group D after treatment of HCV and its level in HCC patients unlike the condition before treatment. Levels of MiRNA-122 in patients of group C decreased after treatment of HCV. MicroRNA-122 was lower in HCC group B than group C after treatment of HCV although it showed significantly higher levels before treatment.

Patients with late fibrosis stages showed a greater reduction in levels of MiRNA-122than patients with early hepatic fibrosis (P=0.008) after treatment of HCV.

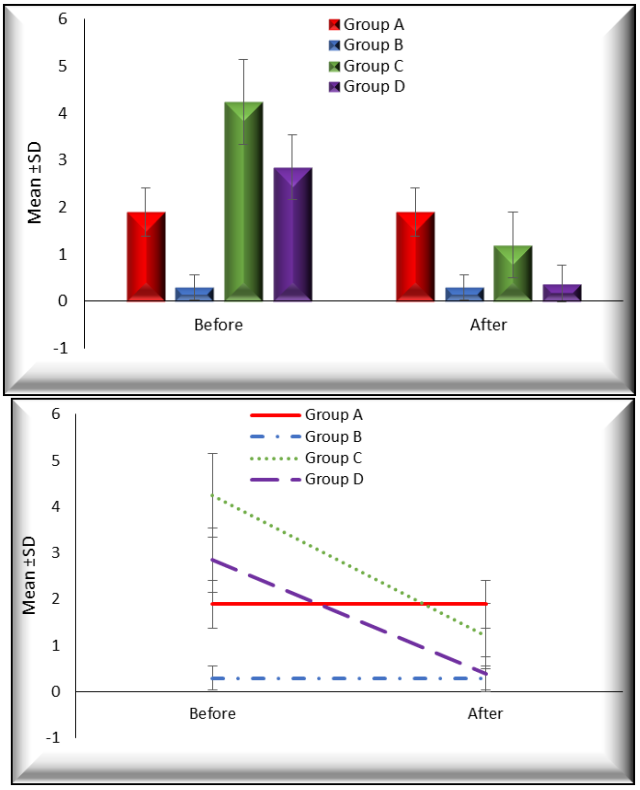


Figure 1: Levels of MiRNA -122 before and after treatment of HCV in infected groups C and D

Table (2): Comparison of microRNA -122 level between groups A &B and HCV group (both C + D groups)

microRNA 122		Groups			ANOVA	
		A (n = 10)	B (n = 10)	(C+D) HCV (n = 20)	F	P
Baseline	Range	1.23 - 2.53	0.103 - 0.743	1.75 - 5.53	27.601	<0.001*
	Mean ±SD	1.890 ± 0.514	0.294 ± 0.259	3.544 ± 1.061		
After end of HCV therapy	Range	-	-	0.023 - 2.345	9.006	0.001*
	Mean ±SD	-	-	0.787 ± 0.694		
Differences	Mean ±SD	-	-	2.757 ± 0.811		
Paired T Test	P-value	-	-	<0.001*		

TUKEY'S Test			
	A&B	A&X	B&X
Before	0.007*	0.003*	<0.001*
After	-	0.004*	0.269

Baseline microRNA-122 level was significantly lower in (HCC) group B than other groups. It was also significantly higher in HCV groups than group A. After end of HCV therapy, microRNA- 122 was significantly lower in HCV groups than healthy group A. But it showed no statistically significant difference with HCC group B.

MicroRNA-122 was significantly lower after completion of HCV therapy than before treatment in HCV groups.

Table (3): Correlations between microRNA122 change and other variables in groups C&D

	microRNA 122 Change			
	Group C		Group D	
	R	P	R	P
Age	0.556	0.095	0.260	0.469
BMI	-0.148	0.684	0.180	0.619
ALT (IU/L)	-0.530	0.115	0.426	0.219
AST (IU/L)	-0.818	0.053	0.231	0.521
AFP (IU/L)	0.166	0.646	-0.214	0.552
Albumin (g/dL)	-0.336	0.342	0.181	0.616
Total Bilirubin (mg/dL)	0.368	0.295	0.207	0.567
Indirect Bilirubin (mg/dL)	0.490	0.151	0.227	0.529
S. Creatinine	-0.200	0.579	0.167	0.645
INR	-0.360	0.307	0.063	0.862
HB (g/dl)	0.251	0.484	0.272	0.447
WBC(x10^3/mm^3)	-0.191	0.598	-0.592	0.072
ANC(x10^3/mm^3)	0.144	0.692	-0.896	0.051
Platelets(x10^3/mm^3)	0.207	0.566	-0.443	0.200
Fib4 Calculation	0.144	0.692	0.404	0.247
PCR onset of therapy	0.101	0.782	0.316	0.373

There was no significant correlation between microRNA 122 change in groups C & D and other variables.

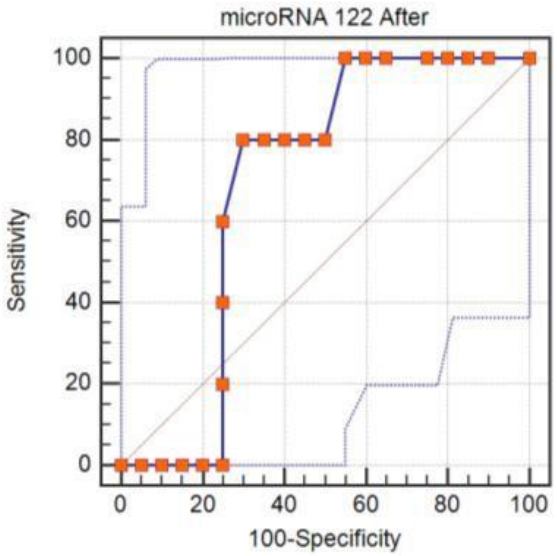
Table (4): Correlations between baseline microRNA-122 level and other variables in group B

Correlations		
	baseline microRNA 122	
	Group B	
	r	**P-value**
Age	0.574	0.311
BMI	0.315	0.606
ALT (IU/L)	-0.212	0.733
AST (IU/L)	-0.580	0.306
AFP (IU/L)	-0.654	0.231
Albumin (g/dL)	0.499	0.392
Total Bilirubin (mg/dL)	-0.819	0.090
Indirect Bilirubin (mg/dL)	-0.592	0.293
S. Creatinine	-0.653	0.232
INR	-0.456	0.440
HB	-0.450	0.447
WBC(x10^3/mm^3)	0.682	0.205
ANC(x10^3/mm^3)	0.755	0.140
Platelets(x10^3/mm^3)	-0.659	0.226
Fib4 Calculation	0.027	0.965

There was no significant correlation between microRNA- 122 level in HCC group B and other variables.

Table (5) and figure (2): ROC Curve for microRNA-122 regarding a cut-off value of HCC

ROC curve between Group B and Group C&D						
	Cutoff	**Sens.**	**Spec.**	**PPV**	**NPV**	**Accuracy**
microRNA 122	≤0.26	80.0	70.0	40.0	93.3	69%



DISCUSSION

Hepatocellular carcinoma considered as very potential health burdens in developing countries as Egypt because of higher prevalence of HCV infections compared to developed countries (22).

The most common triggering agents were chronic viral hepatitis and cirrhosis. HCV in Egypt is highest rate in worldwide (23). HCC always runs fulminant course with bad prognosis (5). Therefore, the purpose of surveillance for HCC is to recognize tumors early when they might be more amenable to curative therapy and hence decrease HCC- related mortality (24).

MicroRNAs controlled of genes expression at post-transcriptional levels and play crucial roles in cancer developing which related to its acting as oncogenes or tumor suppressors (25). Moreover, because it modulates HCC function, which might have therapeutic effects as targeted molecular therapy (26). MiR-122 levels are commonly lowered in HCC comparing with normal liver (10).

miR-122 overexpressions decrease the tumorigenic property in HCC cell, which could be related to its roles as tumor suppressor gene, and improving cellular responses to chemotherapeutic drugs sorafenib and doxorubicin (10).

Many studies tried to assess the relationship between treatment of HCV by DAAs and HCC, mostly suspecting that HCV therapy by DAAs may increase risk of HCC.

In the present study we evaluated microRNA-122 level at end of HCV treatment by DAAs and compared the results with micoRNA -122 level in HCC patients trying to include or exclude its possible role in carcinogenesis.

HCC recurrences risks are widespread regardless of treatment modality and preventing remains unmet issue by HCV eradication with DAAs which might be new option (27).

There is a risk of recurrence in patients with a history of HCC that may be associated with intrinsic tumour factors and underlined liver disease, such as active viremia and degree of liver function. Our hypothesized is HCV eradication induce by DAAs outcome in reducing immune surveillance dysregulating the anti-tumor responses and growing still undetected microscopic HCC tumor clones. On the other hand, effective antiviral therapy can lead to regression of fibrosis and improvements in portal hypertension and liver functions. In this context, DAAs will reduce the risk of recurrence of HCC by eradicating HCV and enhancing liver function (28).

In the current study, the mean age of patients in HCC group B was (63.6±6.2) years. In Egypt, after the successful campaign for treatment of chronic hepatitis C with DAAs in 2015follow up and screening for HCC are exceedingly important (29).

In the studied population, HCC is commonly diagnosed in males more than females (60%, 40%) respectively with male to female ratio 3:2 as previously shown in similar studies (30,31).

HCC in Egyptian is 1st prevalent malignancy in men, 2nd prevalent in women. HCC incidence increase to19.7% of the total cancer cases in 2018. Rising HCC incidence might due to increasing HCV frequency and complications, advancing in screen programs and diagnostic (32). Globally, HCC high prevalence in men may be explained by differences in exposure to risk factors especially viral hepatitis among men. Sex hormones and other x-linked genetic could have important effects as estrogens and androgens which might modulate hepato-carcinogenesis and given explanations for increasing HCC incidence in men (33).

In this study, mean of Baseline miR-122 level (0.294±0.259) was significantly lower in HCC group B than other groups ($p<0.001$) as in table (1). This finding comes in agreement with Amr et al. in 2017(34), Weis et al in 2019(35) and El-Garem et al. in 2014(36). Which could explained be miR-122 might down-regulate target mRNA of obscure tumor suppressor genes and prompts tumor developing (37).

In a paper by El-Abd et al. in 2015 no statistical difference was found when comparing levels of miR-122 among HCC cases and other without HCC. Reduced levels in HCC cases were reported while hadn't reached significantly (40). They attributed the discrepancy between their findings and other studies to the marked variability in the incidence of HCC among different populations.
Increased miR-122 expressions in HCV cases comparing with control (table 1). This was observed in some studies and was explained by the possible miR-122 leakage from apoptotic cells into the blood (34,41).
9 HCC-related miRs evaluated in HCV cases, HCC patients and health control subjects. median serum levels of 7 out of 9 evaluated miRs significant low in HCC cases (42).
Liver damage led to increasing miR-122 in serum and decreased with developing fibrosis (43).

In this investigation, mean level of miR-122 at end of HCV therapy was significantly lower in advanced liver fibrosis cases (F3/F4) than cases with early fibrosis (F1/F2) and healthy controls ($P<0.001$). However, level of MiRNA -122 was of no notable difference between HCC and advanced fibrosis groups. This implies similar status of miR-122 in late fibrosis (after treatment of HCV) and HCC which might suggest a possible link between HCC development and advanced liver fibrosis after DAA therapy for HCV. This is similar to previously mentioned studies linking development of HCC to presence of cirrhosis in HCV infected patients.

At end of HCV therapy, miR-122 was significantly lower in HCV infected patients than healthy controls ($p=0.004$) as in table (2) but levels were comparable to patient with HCC ($p=0.269$). Whether this reduction is pathologically related to HCC development is still questionable.

MiR-122 level at end of HCV therapy in all HCV infected patients collectively was lower than the level before HCV treatment with statistical significance ($p<0.001$). This agrees with the study by Waring et al. (2015) who found an average four folds reduction in miR-122 between baseline and post treatment week 12 in subjects who achieved SVR although that study used a different treatment regimen for HCV (paritaprevir/ritonavir + dasabuvir + RBV for 12 weeks, or paritaprevir/ritonavir + ombitasvir ± RBV for 12 weeks) (44).

These findings may indicate a correlation between treatment of HCV and development of HCC. Further studies in this rich area of research are needed to confirm or refute our results due to the

small sample size in our study. Moreover, the investigation of other miRNAs and altered in HCV and HCC which might be valuable addition to our observation. A study of the sensitivity and specificity of many miRNAs in HCV and HCC cases showed that median serum level of seven miRNAs was significantly lowered in HCC patients whereas others were reduced in HCV compared to healthy controls (42).

In this study, correlation among miR-122 levels and clinical and laboratory parameters in HCV or HCC cases were not significantly differ (tables 3, 4) although others reported there were correlation among miR-122 and higher ALT and AST levels in chronic hepatitis groups (34 and 36).

The lack of correlation of miR-122 and other laboratory parameters may signify that it is an independent predictor of HCC development in HCV related cirrhosis if it decreases after HCV eradication, however further studies on a large number of patients are needed. Our results are suggestive that miR-122 is an independent parameter in evaluation of cirrhotic patients after antiviral therapy and is an independent parameter to assess its possible relation to carcinogenesis in cirrhotic patients.

miR-122 (ROC analysis) in discriminated HCC cases from chronic HCV had AUC of 0.69 with sensitivity (80%) specificity (70%_, positive predictive value (PPV) (40, negative predictive value (NPV) (93.3) at cutoff value ≤0.26 (RQ) as figure (2), which in agree Amr et al. in 2017.
In summary, available results were not consistent enough for judging on HCC occurrence/recurrence risks of post IFN-free HCV eradication treatment most be taken into account. Other postulated molecular mechanisms of HCC development after treatment by DAAs including disturbed growth factor as VEGF and disturbed immune function after viral eradication and epigenetic effect of HCV infection that may help carcinogenesis should be considered (27).

In Conclusion, MiR-122 was significantly reduced at end of HCV therapy with DAAs and became similar to values in HCC patients. Whether this observed reduction is mechanistically related to hepatocarcinogenesis is still a possibility, to be clarified in future large scale studies.
Baseline MiR-122 level at cutoff value ≤0.26 was significantly lower in HCC patients than chronic HCV patients and normal controls, with a sensitivity of 80%, a specificity of 70%. The reduction of MiR-122 at the end of HCV therapy with DAAs was significantly observed in (F3, F4) patients than those with early fibrosis stages (F1, F2). This again gives a possible explanation of HCC development in HCV patients with advanced fibrosis (cirrhosis) and raises the question about the diagnostic and therapeutic value of miRNA 122 (and possibly other miRNAs) in the management strategy of HCV infected patients.

The diagnostic and possible therapeutic value of miRNA 122 in HCV infected patients needs to be subsequently investigated.

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Compliance with Ethical Standards

The ethical committee in Ain shams University and the Department of internal medicine, Ain shams University approved this study and it has been performed according to Declaration of Helsinki ethical standards. All participants included in the study provided informed consent before enrollment.

Conflict of Interest

We haven't any conflict of interest

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