RESEARCH ARTICLE

Serum miRNA Panel in Egyptian Patients with Chronic Hepatitis C Related Hepatocellular Carcinoma

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Abstract

Background: Primary hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. MicroRNAs (miRNAs) have great HCC diagnostic potential and circulating miRNAs have been reported as promising biomarkers for various pathologic conditions. Aim: To explore the potential benefit of serum miR-126, miR-129, miR-155, miR-203 and miR-223 as non-invasive diagnostic markers of hepatitis C virus (HCV)-related HCC. Materials and Methods: The expression of miRNA was evaluated using real-time quantitative RT-PCR in 78 serum samples (30 treatment-naïve chronic HCV, 25 post-HCV compensated cirrhosis and 23 treatment-naïve HCC cases). Results: Comparing miRNA fold changes in the HCC group vs the non HCC groups, there was significant fold decrease in miR-126 (P= 0.034), miR-129 (P= 0.006), miR-155 (P= 0.011), miR-203 (P<0.001) and miR-223 (P= 0.013). The highest AUC to differentiate HCC patients from non-HCC was 0.76 for miR-203. Conclusions: Among studied miRNAs, serum miR-203 has the highest potential as a non-invasive biomarker of HCC.

Keywords: HCC diagnosis - chronic HCV infection - serum miRNAs - Egypt

Introduction

The hepatocellular carcinoma (HCC) is a common disorder worldwide with a median survival of 6-8 months and ranks 2nd and 6th most common cancer among men and women in Egypt, respectively (Omar et al., 2013). HCC has a rising incidence in Egypt mostly due to high prevalence of viral hepatitis C (Anwar et al., 2008). Surveillance of at risk populations may detect tumors at an early stage where curative interventions can be implemented. The performance of available serum biomarkers in early diagnosis of HCC is sub-optimal. Alpha-fetoprotein (AFP) is widely used to detect primary HCC, whereas its sensitivity and specificity are not satisfying. Thus, the early diagnosis of hepatocellular carcinoma is still challenging and requires specific biomarkers.

miRNA is a noncoding RNA gene product that controls gene expression by altering the stability or translational efficiency of its target miRNAs. MiRNAs have been reported to be aberrantly present in neoplastic cells compared with their normal counterparts. Moreover, miRNAs are detectable and stable in serum. Several studies examined the miRNAs expression profile in different types/stages of human liver cancers and noncancerous specimens (Chen, 2009; Huang and He, 2011; Borel et al., 2012; El-Abd et al., 2015). However, data about serum miRNAs profiling among Egyptian patients are still limited and warrants further work up.

Materials and Methods

Patients

During the period between March and June 2012 serum samples were collected from 78 consecutive HCV-infected patients presenting to tropical medicine department, Kasr Alainy hospital: 30 non-cirrhotic chronic HCV, 25 with HCV-related cirrhosis and 23 with HCV-related HCC (diagnosed according to EASL guidelines 2012). Serum samples were also collected from 10 age and sex-matched healthy volunteers (defined as those with normal transaminases, normal hepatic ultrasound and negative for HBsAg, HBc-Ab and HCV Ab). All patients were recruited after a written informed consent and the study protocol was approved by the ethics review committee of Cairo University hospital. Exclusion criteria included: patients with positive HBsAg or with history of other causes of chronic liver disease other than HCV, previous treatment for HCC or antiviral therapy for HCV and any associated malignancies other than HCC.

RNA extraction

For the real-time PCR, RNAs were extracted from serum using TRIzol according to the manufacturer's instruction. The RNA purity was assessed by the RNA concentration and quantified by NanoDrop ND-1000 (Nanodrop, United States). Single-stranded cDNAs were generated using the RT kit (Qiagen, Valencia, CA,

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United States) according to the manufacturer's directions (miSCript miRNA PCR system, miRneasy mini kit for miRna extraction, miScript RT II for miRna reverse transcription, miSCript Primer Assay and miSCript SYBR Green PCR Kit for PCR amplification.

RNA quantification

PCR quantification experiments were performed with PCR (Applied Biosystems; Foster City, CA) using the SYBR Green PCR Master Mix according to the manufacturer's protocol. The primers for microRNA-122, -221 and housekeeping gene were supplied by Qiagene, Germany (catalog numbers 3416, 3857 and 33712). The housekeeping miRNA SNORD68 was used as the endogenous control. Fluorescence measurements were made in every cycle and the cycling conditions used were: 95°C for 30 s, and 40 cycles of 95°C for 5 s and 60°C for 34 s.

Expression of miRNAs was reported as ΔCt value. The ΔCt was calculated by subtracting the Ct values of miRNA SNORD68 from the Ct values of the target miRNAs. As there is an inverse correlation between ΔCt and miRNA expression level, lower ΔCt values were associated with increased miRNA. The resulting normalized ΔCt values were used in calculating relative expression values by using 2-Δ(Ct), these values are directly related to the miRNA expression levels. The 2-ΔΔ (Ct) method was used to determine relative-quantitative levels.

Statistical analysis

Patients were categorized into 3 groups; chronic HCV, cirrhosis and HCC. Further comparisons were performed between HCC group and Non-HCC (chronic HCV and cirrhosis). Quantitative variables were expressed by mean ± SD or expressed by median and inter quartile range (IQR) for non-parametric data. They were compared by t-student or ANOVA test when appropriate. Qualitative variables were compared by χ^2 or Fischer's exact test when appropriate. Receiver operator characteristic (ROC) curves were constructed to assess the value of miRNA in diagnosing HCC and to assess area under the curve (AUROC). AUROC less than 0.60 with P value >0.05 is considered unreliable for ROC curve. Spearmen and Pearson correlations were done for correlating quantitative variables. In all tests, P value was considered significant if less than 0.05.

Results

The demographic parameters of the studied participants are shown in Table 1. There was a significant difference between the diseased groups regarding age (p value<0.001). Regarding gender difference; males were predominant in diseased groups and they represented 87%, 68%, 73.3% in HCC, cirrhosis and chronic HCV groups respectively with no statistically significant difference between the studied groups (P=0.29). HCC related characteristics (n=23) are described in Table 2.

Differential expression of serum miRNA levels in HCC patients

Comparing HCC group to non HCC group, serum miR-126 (P= 0.034), miR-129 (P= 0.006), miR-155(P=

Table 2. Tumor-Related Characteristics (n = 30)

Parameter		Number (%)
AFP level (0-10)	Normal	4 (13.4)
	Elevated	26 (86.6)
Child score	A	12 (52)
	B	9 (39)
	C	2 (9)
Performance status (PS)	PS 0	24 (80)
	PS 1-2	4 (13.4)
	PS > 2	2(6)
BCLC	Stage 0	0 (0)
	Stage A	1(3.8)
	Stage B	19 (73.1)
	Stage C	4 (15.4)
	Stage D	2 (7.7)
Number of focal lesions	Single	17 (56.7)
	Multiple	13 (43.4)
Site of focal lesions	Right lobe	18 (60)
	Left lobe	5 (16.7)
	Both	7 (23.3)
Tumor size by CT	< 3 cm	1 (3.3)
	3-5 cm	12 (40)
	> 5 cm	17 (56.7)
Portal vein invasion	Yes	7 (23.3)
	No	23 (76.7)

Table 1. Demographic and Clinical Parameters of Patients

Variable	HCV	cirrhosis	HCC	P value
Age (yr), Mean ± SD	38.20±8.21[A]	55.52±7.32[B]	59.45±8.03[B]	< 0.001
Gender (male), n (%)	22 (73.3%)	17(68%)	20(87%)	0.29
Hb (gm/dL)	14.23±1.58[B]	10.54±2.15[A]	11.20±3.16[A]	< 0.001
TLC × 103/mm³	6.15±2.13[A]	7.00±4.03[A]	6.07±2.75[A]	0.488
PLT× 103/mm³	228.80±59.74[B]	113.13±73.84[A]	133.26±80.18[A]	< 0.001
AST (0-37 IU/L)	94.43±70.34[B]	57.76±30.63[A]	113.39±49.36[B]	0.002
ALT (0-42 IU/L)	67.23±37.67[B]	31.84±23.26[A]	61.30±41.52[B]	0.001
ALP (0-129 IU/L)	89.60±37.30[A]	149.04±92.04A[B]	186.71±131.26[B]	0.001
BIL T (mg/dL)	0.74±0.26[A]	4.26±7.32[B]	2.12±2.25[AB]	0.014
BIL D (mg/dL)	0.17±0.14[A]	2.83±6.06[B]	1.05±1.58[AB]	0.024
Albumin (g/dL)	4.22±0.36[C]	2.53±0.59[A]	3.11±0.38[B]	< 0.001
Creatinine (mg/dL)	0.80±0.24[A]	1.19±0.56A[B]	1.43±1.37[B]	0.023
PC (%)	88.20±10.94[C]	52.48±18.51[A]	68.65±17.37[B]	< 0.001
AFP log10 (ng/dl)	0.57±.39[A]	0.88±.52[A]	2.58±1.17[B]	< 0.001

[A,B,C] Groups with different letters show significant difference, those with similar letters show no significant difference. HCC: Hepatocellular carcinoma.

Table 3. Serum miRNAs in HCC and Non HCC Groups

Parameter		HCC (n=23)	Non-HCC (n=55)	P value
mi R-126	Mean ± SD	3.47±3.75	7.60±17.73	0.034
	Median	1.59	0.56	
miR-129	Mean ± SD	4.96±3.64	9.77±20.52	0.006
	Median	4.16	0.7	
miR-155	Mean ± SD	5.20±2.85	9.72±20.23	0.011
	Median	5.21	0.93	
miR-203	Mean ± SD	5.47±3.64	8.73±20.21	<0.001
	Median	4.12	0.78	
miR-223	Mean ± SD	7.23±4.26	10.34±19.52	0.013
	Median	6.95	1.2	

Table 4. Correlation of Studied Serum miRNAs with Other Parameters in the HCC Group

| Parameter		mi R-126	miR-129	miR-155	miR-203	miR-223
Age	R	0.01	-0.03	0.08	0.5	0.08
	P	0.97	0.88	0.74	0.02	0.72
Hb	R	0.02	-0.18	-0.04	-0.22	0.08
	P	0.95	0.41	0.84	0.32	0.72
TLC	R	-0.29	-0.13	-0.36	-0.15	-0.29
	P	0.17	0.55	0.09	0.5	0.18
PLT	R	-0.23	-0.09	-0.14	-0.17	-0.15
	P	0.28	0.67	0.53	0.44	0.51
AST	R	0.09	0.12	0.24	-0.1	-0.19
	P	0.67	0.6	0.26	0.65	0.39
ALT	R	0.13	0.08	0.42	0.01	-0.09
	P	0.54	0.73	0.04	0.98	0.68
ALP	R	-0.07	0.16	0.08	-0.46	0.03
	P	0.75	0.49	0.72	0.04	0.91
BIL T	R	-0.12	0.25	-0.05	-0.17	0.07
	P	0.59	0.24	0.83	0.43	0.76
BIL D	R	-0.05	0.24	-0.04	-0.05	0.02
	P	0.84	0.28	0.84	0.81	0.94
Albumin	R	-0.29	0.15	0.01	-0.39	-0.19
	P	0.18	0.49	0.97	0.07	0.39
Creatinine	R	-0.04	0.09	-0.12	-0.15	-0.06
	P	0.87	0.69	0.59	0.49	0.78
PC	R	0.15	-0.14	0.11	-0.11	-0.15
	P	0.5	0.53	0.62	0.62	0.48
AFP	R	0.02	-0.27	-0.24	0.13	-0.28
	P	0.94	0.21	0.28	0.54	0.21
child score	R	0.14	0.28	0	0.17	0.12
	P	0.53	0.21	0.99	0.44	0.59
BCLC	R	-0.24	-0.06	-0.18	0	-0.26
	P	0.26	0.77	0.42	0.99	0.24

R: correlation coefficient; P: Pvalue

0.011), miR-203 (P <0.001) and miR-223 (P= 0.013) expression levels were downregulated (Table 3).

Diagnostic performance of studied serum miRNAs

The diagnostic performance for the established miRNA panel was evaluated using ROC analysis. It revealed that studied miRNAs could discriminate between HCC and non HCC subjects (Figure 1) with AUC=0.65 for miR-126 (95% CI 0.54–0.77), 0.68 for miR-155 and miR-223 (95% CI 0.57–0.8 and 0.56-0.8, respectively), 0.7 for miR-129 (95% CI 0.59–0.81) and 0.76 for miR-203 (95% CI 0.65–0.86), respectively.

Correlation of studied serum miRNAs with other parameters in HCC group

Serum miR-203 was positively correlated with age
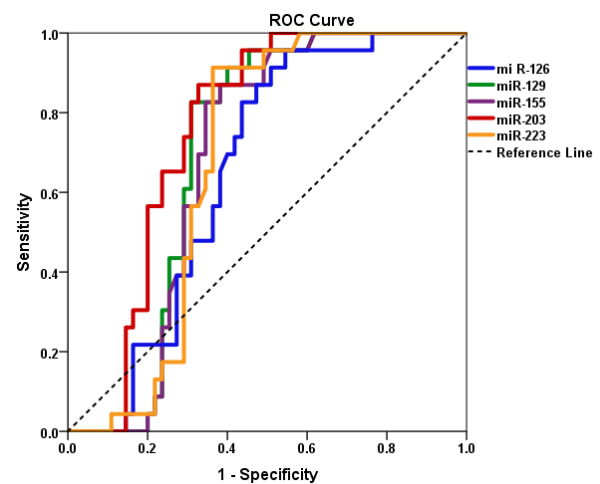
Figure 1. Serum miRNAs in HCC vs non-HCC Groups

and negatively correlated with serum ALP level. Also, miR-155 was positively correlated with serum ALT level. Otherwise, no significant correlation between studied serum miRNA and other parameters was found (Table 4).

Discussion

Currently, the identification of HCC-specific miRNA profiles in the circulation is an emerging field of particular interest. HCC represents an extremely poor prognostic cancer that remains one of the most common and aggressive human malignancies worldwide. Over the last 2 decades, the performance of tumor markers for HCC diagnosis has not been optimal (okuda et al., 2000; Marrero and Lok, 2004). miRNAs have been implicated in roles affecting cellular proliferation and oncogenesis (Bushati and Cohen, 2007). Cellular miRNAs have been linked with HCC. Their availability in the circulation makes them a potential target for early tumor detection (Llovet et al., 2003). The aim of the present study was to study the potential utility of serum miR-126, miR-129, miR-155, miR-203 and miR-223 as noninvasive markers for diagnosis of HCV related hepatocellular carcinoma among Egyptian patients.

HCC patients were within Child-Pugh A and B classifications (52%, 39% respectively), 82% were stage B on BCLC scoring system. This could be explained by the fact that most of them were referred for interventional treatment. Another possible explanation is implementation of surveillance programs; allowing detection of tumors at an early stage in well compensated patients. Moreover, serum AFP level was normal (<10 ng/dL) in 13.4% of recruited HCC patients. Similar finding was observed by Chen et al. (1984) who suggested that not all tumors secrete AFP, and serum levels are normal in up to 40% of small HCCs. Thus, AFP alone is not recommended for the diagnosis of HCC (Tateishi et al., 2008).

In this study, we confirm that some miRNAs can be measured from a relatively small amount of serum. A few studies reported altered levels of circulating miRNAs in association with HCC (Borel et al., 2012).

In our study, analysis of fold changes in expression level of miR-203 showed significant fold decrease in

expression level in HCC group (5.47±3.64) in comparison to non HCC groups (8.73±20.21) (P<0.001, AUC= 0.76). MiR-203 is considered a tumor-suppressive miRNA, contributing to the carcinogenesis and deterioration of HCC. Microvessel density (MVD) showed a significant correlation with miR-203 (r = −0.206, P=0.045). MVD is related to angiogenesis and plays a vital role in tumor growth and progression (Liu et al., 2015). Furuta et al. (2010) suggested that the silencing of miR-203 contributed to the pathogenesis of HCC by activating of ATP binding cassette E1 (ABCE1). It was demonstrated that low expression of miR-203 dedicated to the progression of HCC via targeting survivin, oncogene ADAM9 (a disintegrin and metalloproteinase 9) and oncogenic long non-coding RNA HULC (highly up-regulated in liver cancer) (Wei et al., 2013, Wan et al., 2015). All these findings strongly support our result that low expression of miR-203 was relevant to the development of HCC via different pathways.

Analysis of fold changes in expression level of miR-223 displayed significant fold decrease in expression level in HCC group (7.23) in comparison to non HCC groups (10.34). Serum miR-223 yielded an AUC of 0.68 (95% CI: 0.65–0.79) for discriminating HCC from Non HCC subjects (P value 0.013). MiR-223 is one of the miRNAs that has been given much attention in the literature. This miRNA is usually regarded as a bone marrow specific miRNA that functions as an important modulator of cellular differentiation (Fukao et al., 2007; Johnnidis et al., 2008). A study observed that miR-223 was commonly repressed in HCC cells. MiR-223 participates in HCC pathway thorough regulating the G2/M transition mediated by stathmin-1 (Wong et al., 2008). However, Xu et al. (2011) found that serum miR-223 were significantly higher in patients with HCC than those in healthy controls with no significant difference between HCC and chronic hepatitis patients. This apparent difference with ours could be explained as we compared HCC to non HCC patients (chronic hepatitis and liver cirrhosis) but not to individual group. Elevated serum miR-223 could also come from tissue injury such as hepatitis. Since patients with chronic hepatitis may have more serious damage of hepatocytes than patients with HCC, much higher level of serum miR-223 in patients with chronic B hepatitis than in patients with HCC. Similar results have been obtained in a previous study, showing that elevation of serum miR-223 come from hepatic ischemia/reperfusion injury (Yu et al., 2009).

Interestingly, we found a decrease in expression level of serum miR-129 in HCC patients' (4.96±3.64) in comparison to non HCC patients (9.77±20.52) with P value 0.006 and AUC 0.7. It has been clearly demonstrated that the loss of miR-129 expression (potential tumor suppressor) is significantly associ¬ated with disease progression in colorectal cancer, gastric cancer, and liver carcinoma (Fesler et al., 2014). The epigenetic deregulation of miR-129 thorough hypermethylation has been demonstrated by different groups in hepatocellular carcinoma (HCC) (Lu et al., 2013).

We found that serum miR-126 displayed a significant fold decrease in expression in serum of HCC patients with

P value 0.034 (AUC=0.65). Consistent with this finding, serum miR-126 was reported to inhibit tumorigenesis and to be downregulated in metastasis. It acts mainly thorough targeting VEGF and VCAM-1 (Hung et al., 2014).

In our study, serum miR-155 was significantly (P=0.011) down-regulated in HCC patients compared with non HCC patients with AUC 0.68. Oncogenic miR-155 was significantly up-regulated in HCC and promoted hepatocyte proliferation and tumorigenesis by increasing Wnt signaling (Zhang et al., 2012). High cellular miR-155 expression was an independent predictor of poor prognosis in HCC (Han et al., 2012).

Most studies assessed tissue miRNA rather than serum levels. The different results could also be explained by technical variations including sampling methods and freezing and miRNA isolation procedures. The etiology of liver disease is also variable in different studies including viral and alcoholic. The stage of the disease is also a source of variation especially that it is still not evident how miRNA expression changes with fibrosis progression. Different studies have also used different control samples for normalization, e.g., non-HCC tissue from the same patient, healthy liver tissue from another subject or patients with the same pathology but not HCC (Borel et al., 2012).

Better results could be obtained if combined with other sero-markers and testing a panel of miRNA's collectively could ultimately serve as a reliable diagnostic test for HCC. These promising results should be validated in a larger patient cohort; nevertheless, clinical relevance of serum miRNAs as potential diagnostic, prognostic tools, therapeutic targets and biomarkers of treatment efficacy should be evaluated.

In conclusion, studied serum miRNAs; particularly serum miR-203 could distinguish HCV-related HCC from HCV-associated Liver disease and healthy control subjects suggesting their potential usefulness as HCC biomarkers and clinical utility in diagnosis of HCV-related HCC.

Acknowledgement

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