RESEARCH ARTICLE

Expression of miR-20a: A serum biomarker in the diagnostic approach for hepatocellular carcinoma

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Abstract

Objective: To determine the micro-ribonucleic acid-20a expression in hepatocellular carcinoma patients and its correlation with patients' demographic data, clinico-pathological and staging characteristics.

Methods: The case-control study was conducted from January to December 2014 at Isra University, Hyderabad, and comprised samples of hepatocellular carcinoma patients collected from two hospitals in Karachi and one in Hyderabad. Patients infected with chronic hepatitis B and C infections formed group-I and group-II and were compared with healthy controls in group-III. SPSS 21 was used for data analysis.

Results: There were 225 subjects divided into three equal groups of 75(33.3%) each. Among the controls, 57(76.0%) showed up-regulation, whereas 66(88.0%) in group-I and 50(66.7%) in group-II showed decreased expression. Micro-ribonucleic acid-20a showed significant down-regulation in group-I compared to group-II ($p<0.001$). Significant correlation of the down-regulation was found with male gender, age below 50 years and alpha-fetoprotein level below 20ng/ml ($p<0.05$).

Conclusion: Micro-ribonucleic acid-20a expression was found to be decreased in hepatocellular carcinoma patients with significant correlation with gender, age and alpha-fetoprotein level.

Keywords: Hepatocellular carcinoma, Micro RNA, Child Pugh score, Viral hepatitis. (JPMA 69: 29; 2019)

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer with over half-a-million new cases being diagnosed annually worldwide.[1] Hepatitis B and C viruses (HBV and HCV) contribute approximately 80% of HCC cases. In geographical terms, the incidence of HBV infection in China is over 40% and eastern Asia and sub-Saharan Africa are over 80%.[2,3]

Currently, computed tomography (CT), ultrasonography (US), magnetic resonance imaging (MRI) and alpha-fetoprotein (AFP) levels are generally used to diagnose HCC. Serum AFP is effective and simple test for screening HCC, but accurate diagnosis is problematic in almost one-third of HCC patients with low AFP levels.[4,5]

Many of the micro-ribonucleic acid (miRNAs) have been reported to be down-regulated and others are over-expressed in progressive liver fibrosis and HCC patients.[6,7] The miR-20a gene showed decreased expression in many tumours, including carcinoma of breast and pancreas, while at the same time over-expression was seen in adenocarcinoma of large intestine and brain tumours.[8] Expression of miR-20a was recognised as expected biomarker for HCV-associated liver carcinoma.[9] It showed further decreased expression in post-liver transplantation HCC patients.[10]

The current study was planned to determine the expression of miR-20a in HCC patients and its correlation with patients' demographic data, clinico-pathological and staging characteristics. The null hypothesis was that miR-20a expression was not affected in HCC patients.

Materials and Methods

The case-control study was conducted from January to December 2014 at Isra University, Hyderabad, and comprised samples of HCC patients collected from Jinnah Postgraduate Medical Centre, Karachi, Asian Institute of Medical Sciences, Hyderabad, and Civil Hospital, Karachi. After approval from the institutional ethics committee, HCC patients were divided into equal groups, with group-I comprising those infected with HBV, and group-II comprised those with HCV Healthy individuals formed the control group-III. The sample size was calculated as per sampling formula and incidence of HCC was taken at 5%.[11,12] The blood samples of patients with known causes of chronic hepatitis other than HBV or HCV or co-infection with either of them and patients who received treatment for either HCV or HBV were excluded.

Demographic data, clinical history, laboratory findings including α-fetoprotein and fibroscan were taken from the patient's hospital record on a pre-designed proforma.[13] The Child Pugh score was taken according to

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Pugh RNH et al study and Barcelona Clinic Liver Cancer staging was done according to Llovet JM et al and Vitale A et al study.[14-16] Extraction of total ribonucleic acid (RNA) was done by homogenisation, separation, RNA precipitation, RNA washing, and, finally, rehydration of RNA was carried out by TRIzol LS Reagent (RNA Purification Kit).[17] Afterwards, synthesis of complementary deoxyribonucleic acid (cDNA) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward and reverse primers as internal control with Random hexamer primers was done. The realtime polymerase chain reaction (PCR) was carried out for miR-20a expression using specifically-designed primers of miR-20a;[18] miR-20a forward 5'-GCACTAAAGTGCTTATAGTGCAG- 3'; miR-20a reverse 5'-GTACTTTAAGTGCTCATAATGCA- 3'; and 5S forward and 5S reverse primers as PCR internal controls.

SPSS 21 was used for data analysis. Chi-square test was applied as cross-tabulation for comparison of data among the three groups. Sigmoid curves showing miRNAs' expression were taken by Bio-Rad CFX software, whereas graph was presented with Microsoft excel.[18] Formi RNA correlation with clinico-pathological data, odds ratio (OR) with 95% confidence interval (CI) was done. Statistical significance was taken at $p \leq 0.05$.

Results

There were 225 subjects divided into three equal groups of 75(33.3%) each. In group-III, 57(76.0%) samples showed up-regulation, whereas in 66(88.0%) in group-I and 50(66.7%) in group-II showed decreased expression. Group-I showed significant miR-20a down-regulation compared to group-II (p<0.001) (Figures-1,2).

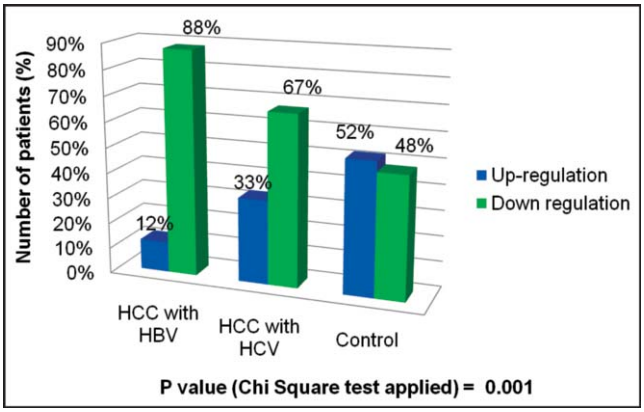

Figure-1: Down-regulation of miR-20a in hepatocellular carcinoma (HCC) patients and up-regulation in controls (n=225).

More down-regulation was seen in the samples of males compared to females (p<0.001). Highest down-regulation was seen in 49(100%) patients aged 50-59 years and 49(81.7%) aged 40-49 years (p<0.001).

Decreased expression of miR-20a was seen in 44(63.8%) samples with AFP below 20(ng/ml), followed by 51(85.0%) with AFP above 200(ng/ml) and all 21(100%) patients with AFP 20-200(ng/ml) (p<0.008.The correlation of fibroscan in HCC patients with miR-20a revealed down-regulation in 5(71.4%) patients with stage F0-F1, 94(75.8%) with stage F2-F3 and 17(89.5%) with stage F4 (p=0.19) (Table-1).

Regarding the correlation of miR-20a expression with

Table-1: Correlation of micro-ribonucleic acid-20a (miR-20a) expression with demographic and clinical findings.

Variable	miR-20a		Univariate analysis		
	Up	Down	P-value	Odds Ratio	(95% CI)
Gender					
Male (n=83)	10(12%)	73(88%)	0.001	2.074	1.780-3.329
Female (n=67)	24(35.8%)	43(64.2%)			
Age					
< 40 years (n=23)	13(56.5%)	10(43.5%)	0.05	1.943	0.980-2.704
40-49 years (n=60)	11(18.3%)	49(81.7%)	0.001	2.008	1.901-3.564
50-59 years (n=49)	0(0%)	49(100%)	0.08	0.761	0.601-1.288
> 60 years (n=18)	10(55.6%)	8(44.4%)			
α-Fetoprotein (ng/ml)					
< 20 (n=69)	25(36.2%)	44(63.8%)	0.008	1.311	0.137-2.002
20-200 (n=21)	0(0%)	21(100%)	0.05	0.774	0.100-1.702
>200 (n=60)	9(15%)	51(85%)			
Imaging findings (Fibroscan)					
F0-F1 (n=7)	2(28.6%)	5(71.4%)	0.29	1.251	0.033-1.915
F2-F3 (n=124)	30(24.2%)	94(75.8%)	0.19	1.221	0.754-3.415
F4 (n=19)	2(10.5%)	17(89.5%)			

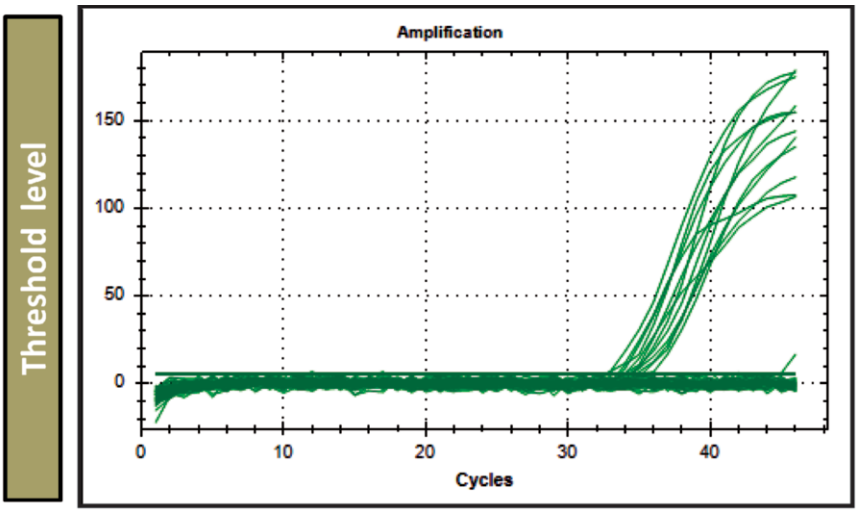

Figure-2: RT PCR curves* showing micro-ribonucleic acid-20a (miR-20a) expression in HCC patients and controls. *Straight lines below the threshold level showing down regulation in patients with hepatocellular carcinoma (HCC) and sigmoid curves showing up-regulation in healthy controls.

size 2-5cm and 40(69%) patients with tumour size greater than 5cm (p=0.06).

Decreased expression was seen in 104(75.9%) patients without metastasis, 9(90%) with nodal metastasis and no patient with distant metastasis (p=0.07).

Decreased expression was seen in 62(69.7%) patients in Child Pugh score A, 49(90.7%) in score B and 5(71.4%) in score C (p=0.15).

Decreased expression was seen in all the 21(100%) cases of Barcelona Clinic Liver Cancer (BCLC) stage O, while 33(68.8%) cases of BCLC stage A, 39(81.2%) cases of BCLC stage B, 18(69.2%) cases of BCLC stage C and 5(71.4%) cases of BCLC stage D showed down-regulation

Table-2: Correlation of micro-ribonucleic acid-20a (miR-20a) expression with clinicopathological and staging characteristics.

Variable	miR-20a		Univariate analysis		
	Up	Down	P-value	Odds Ratio	(95% CI)
Tumour Distribution					
Solitary (n=99)	19(19.2%)	80(80.8%)	0.20	0.608	0.279-1.321
Multifocal (n=51)	15(29.4%)	36(70.6%)			
Tumour Size (cm)					
< 2 cm (n=36)	5(13.9%)	31(86.1%)	0.06	1.790	0.932-3.006
2-5 cm (n=56)	11(19.6%)	45(80.4%)	0.16	0.841	0.777-1.662
> 5 cm (n=58)	18(31%)	40(69%)			
Tumour metastasis					
Absent (n=137)	33(24.1%)	104(75.9%)	0.07	1.005	0.478-2.154
Nodal metastasis (n=10)	1(10%)	9(90%)	0.25	0.747	0.455-1.778
Distant metastasis (n=3)	3(100%)	0(0%)			
Child Pugh score					
A (n=89)	27(30.3%)	62(69.7%)	0.47	0.919	0.168-2.14
B (n=54)	5(9.3%)	49(90.7%)	0.15	1.245	0.781-2.457
C (n=7)	2(28.6%)	5(71.4%)			
BCLC (Barcelona Clinic Liver Cancer) Stage					
O (Very early) (n=21)	0(0%)	21(100%)	0.08	1.057	0.764-2.157
A (Early) (n=48)	15(31.2%)	33(68.8%)	0.12	1.524	0.963-3.246
B (Intermediate) (n=48)	9(18.8%)	39(81.2%)	0.51	0.856	0.349-1.951
C (Advanced) (n=26)	8(30.8%)	18(69.2%)	0.14	0.761	0.347-1.145
D (Terminal) (n=7)	2(28.6%)	5(71.4%)			

tumour distribution 80(80.8%) patients presenting with solitary tumour and 36(70.6%) patients with multifocal tumour revealed decreased expression. Odd ratio and 95% confidence interval showed no correlation with P-value 0.20 (shown in Table-2).

Decreased expression in 31(86.1%) patients with smaller tumour of 2cm or less, 45(80.4%) patients with tumour (p=0.08) (Table-2).

Discussion

The present study was concerned with the expression of miR-20a in HCC patients with chronic HBV and HCV infections in comparison with healthy controls. Data related to miR-20a expression showed 57(76%) subjects in

group-III had up-regulation compared to down-regulation in 88% in group-I and 67% group-II patients. Expression of miR-20a showed significant down-regulation in HBV-associated HCC in contrast to the HCV-associated HCC patients. A study[19] reported the expression of miR-20a in HCC patients and healthy individuals using quantitative PCR (qPCR). In line with our findings, down-regulation was seen in HCC tissue samples. In addition, miR-20a expression after liver transplantation was further decreased in cases of HCC.[20] In HCV-infected patients having evidence of fibrosis, up-regulation was noted when compared with chronic hepatitis patients without HCV infection and healthy controls. It is also reported that miR-20a expression was significantly increased with the increased stages of fibrosis from F1 to F4.[9] Another study reported that miR-20a is also used as therapeutic agent in treating HCC cases as it is also involved in the radio-resistance of malignant hepatocytes.[21] In line with our findings, a study[9] reported the possible role of miR-20a as non-invasive serum biomarker in HCC patients, but, in contrast to our findings, it was significantly correlated more with HCV-positive liver fibrosis in HCC patients than the HBV-related HCC patients. Similarly, in another study, down-regulation of miR-20a was seen in HCC patients in comparison with the normal liver tissues, but after the tumour recurrence in transplanted patients, it was further down-regulated. Therefore survival of the patients was seen poor in HCC patients with down-regulation of miR-20a. Therefore, miR-20a was found to be the best independent predictor which has bad prognosis when using multivariate analysis. Based on the findings, it was suggested that miR-20a can be used as an important therapeutic biomarker for defining the survival of HCC patients.[19]

Our findings showed miR-20a had a male predominance. A study[22] reported down-regulation of miR-20a in male patients with gastric carcinoma. Regarding the age distribution, down-regulation of miR-20a showed correlation between decreased expression and 50-59 years of age. A study[19] reported the mean age of 58 years in patients who showed down-regulation with miR-20a in HCC patients. This is almost in line with the present study. In contrast to our findings, a study[22] reported that miR-20a showed more down-regulation in patients aged over 60 years which might be because of increasing age.

Regarding correlation of AFP level below 20ng/ml, down-regulation was seen in your study similar to previous findings. A study[19] reported that miR-20a correlation with serum AFP levels below 400ng/ml was significantly further reduced in patients with HCC. Down-regulated miR-20a showed no correlation with stage of fibrosis and tumour number whether solitary or multiple in number. In contrast to our findings, expression of miR-20a was decreased significantly with multifocal HCC cases in comparison with the solitary tumour masses.[19]

Regarding the miR-20a expression, it was down-regulated irrespective of the size of primary tumour in HCC patients. One study[22] also reported that down-regulation of miR-20a was correlated with tumour size of less than 5cms. In another study,[19] miR-20a was down-regulated with increasing tumour size in HCC cases which is not in contrast to the findings of the present study.

Also, miR-20a showed no significant association with metastasis. Similarly, a study[22] reported that no significant association of metastasis was noticed with decreased miR-20a expression. This supports the findings of the present study. However, decreased expression of miR-20a was correlated with Child Pugh stage B. The miR-20a showed independent expression of the BCLC stage in the present study.

Conclusion

The miR-20a expression was down-regulated in HCC patients compared to the controls. Decreased expression was particularly more in HBV infected HCC cases. Significant correlation of miR-20a down-regulation was found with gender, age and AFP level. On the contrary, miR-20a correlation with fibroscan, tumour distribution, tumour size, tumour metastasis, Child Pugh score and BCLC stage revealed no association.

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Conflict of Interest: None.

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