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Article

Risk assessment and implications of MicroRNA-210 on hepatocellular carcinoma patients: a case control study

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Abstract. *Background***:** Carcinogenesis in Hepatocellular carcinoma (HCC) is associated with hypoxia and reactive oxygen species (ROS) production. The master hypoxamir, MIRNA-210, is involved in tumor growth, apoptosis, and angiogenesis. MiRNA-210 has been reported to regulate responses and tolerate hypoxia-induced stress. The aim of study is to evaluate circulating miRNA-210 in HCC. *Methods***:** 60 cirrhotic HCC patients (group I) classified into three sub-groups according to BCLC staging (20 patients each); Group (IA): stages 0/A, (IB): stage B and (IC): stages C/D. Group II comprised 60 cirrhotic patients without HCC. Group III included 60 healthy controls. All participants were evaluated clinically, Circulating MIR-210 expression was assessed by real time polymerase chain reaction. *Results***:** MIR-210 was higher in hepatic cancer patients (group I) compared to other groups cirrhotic patients (group II) and normal individuals (group III) ($P < 0.001$ in others). MIR-210 was higher in late HCC patients (sub-group IC) than early and intermediate HCC patients (sub-groups IA and IB respectively) ($P < 0.001$). *Conclusions***:** Significant difference in circulating biomarker MIR-210 between HCC and other groups implies critical role in HCC carcinogenesis.

Keywords: MicroRNAs; liver cancer; biomarkers; hypoxia; Carcinogenesis.

Background

Hepatocellular carcinoma (HCC) ranks as the sixth most common malignancy worldwide, and the 4th in Egypt.(1) It is considered as the third most death-leading cancer.(2) In Egypt, HCC poses a challenging health issue as the burden of HCC patients has raised two folds over the last decade. The liver carcinoma in Egypt counts the first between cancers in males (33.6%) and next to cancer breast between females (13.5%).(1)

The single evident risk factor for HCC development is the liver cirrhosis due to any cause. Liver cirrhosis is present in about 70% to 90% of cases with primary liver cancer. The most prevalent causes of cirrhosis in cases with HCC are chronic infection with one of two viruses, HBV and HCV.(3, 4)

Despite the great advance in clinical therapy, the 5-year survival rate in HCC patients is still not acceptable, mainly due to late diagnosis, multiple cancer metastasis, and high recurrence rates.(5) However, the 5-year survival rate could exceed 70% when cancer patients are diagnosed early.(6)

Until now, combined serum alpha-fetoprotein (AFP) level and abdominal ultrasound are the most commonly used methods for screening of suspicious malignant nodules in cirrhosis. (3) Confirmed diagnosis of HCC is assured by either computed tomography (CT), magnetic resonance imaging (MRI) or biopsy from suspected focal hepatic lesions.(7)

However, high AFP levels don't always indicate HCC, and normal levels don't always rule out HCC. This explains why an AFP test can’t be used on its own to screen for or confirm diagnosis with HCC, and needless to say, it is hence unable to predict the onset and progression of HCC accurately.(8)

It has been demonstrated that the increase in ROS production is initiated by hypoxia, a common feature in cancer. Hypoxia changes the respiratory chain activity, the thing that affects oxidative phosphorylation.(9)

The cell adapts to hypoxic state via increasing expression of certain proteins which promote angiogenesis and erythropoiesis. Consequentially, oxygen availability is raised.(10)

Many hypoxamirs, which are hypoxia-regulated microRNAs (MIRNAs), have been recently specified. The master hypoxamir, MIRNA-210 controls many processes responsible for tumor growth, apoptosis, and angiogenesis.(11)

MiRNA-210 is a unique molecular biomarker engaged in multiple biological activities that happen everywhere the human body. It has multiple functions related to regulation of cell cycle, cellular survival, differentiation, angiogenesis and also metabolism.(12)

MIRNA-210 downregulates several target genes leading to tumor growth and poor prognosis. Generally speaking, from a biological point of view, hypoxamirs can be considered as emerging modifiers of response of cancer cell to the adaptive challenges of the microenvironment. On the other hand, from a clinical perspective, assessing the status of these MIRNAs may help understand the hypoxia-induced mechanisms of resistance.(13)

The aim of the present study is to determine the role of circulating levels of MIR-210 in risk assessment of patients with hepatocellular carcinoma.

Methods

In order to fulfill the aim of the study, we recruited 60 cirrhotic patients with HCC (group I) classified into three sub-groups according to Barcelona clinic liver cancer (BCLC) staging system as follows: Group (IA): 20 HCC patients with BCLC stages 0/A, Group (IB): 20 HCC patients with BCLC stage B and Group (IC): 20 HCC patients with BCLC stages C/D. Group II comprised another 60 patients with liver cirrhosis without HCC (group II). All recruited cirrhotic patients with or without HCC had either hepatitis B virus (HBV), hepatitis C virus (HCV) or mixed infection. Another group of 60 healthy controls (group III) of similar age and sex were also enrolled in the study. All cases of Group I and II were chosen from cases admitted to the Hepatobiliary Unit of the Main Alexandria University Hospital.

The diagnosis of hepatic cirrhosis was confirmed by clinical, ultra-sonographic evidences and/or histopathological examination. Hepatocellular carcinoma diagnosis was determined by the typical vascular fashion of HCC in tri-phasic CT or MRI and examination of surgically-resected tumor samples histopathologically (when available).

Exclusion criteria

Acute and chronic infections other than HBV and HCV infection, chronic alcoholism, presence of other malignancies, presence of significant co-morbidity such as cardiac, respiratory, renal, or connective tissue diseases and patients who had received anti-viral treatment within 6 months or systemic anti-cancer therapy; loco-regional therapy for HCC such as radiofrequency ablation or percutaneous ethanol injection or had trans-catheter arterial embolization before the study.

For the case-control study, the sampling size has been determined considering power 90%, assuming common standard deviation of (18), using F test, with a significance level of 0.05. The sample size was calculated using NCSS 2004 and PASS 2000 program.

All patients and controls were evaluated clinically by taking a full history and thorough clinical examination. This was followed by withdrawal of 10 ml of venous blood from each patient and control in a plain tube. Blood clotting was allowed, then centrifuged at 1200 xg for 10 minutes for serum separation. Serum samples were preserved frozen at -80 °C till the time of the assay.

Measurement of serum MIRNA-210 level using Real-Time PCR and determination of AFP levels using enzyme-linked immunosorbent assay (ELISA) were carried out for all groups.

Determination of serum MIRNA-210 level(11)

This was achieved by total RNA extraction from serum samples. This was done using Qiagen® miRNeasy Mini Kit (Cat. No. 217004). Nano-dropper 2000/2000cc was used for determination of RNA concentration and purity. The next step was quantification of MIRNA-210 expression using the TaqMan MiRNA Assays via two-stage RT-PCR. The first stage was Reverse transcription (RT) in which complementary DNA (cDNA) was reversely transcribed from the purified RNA samples by using specific MIRNA stem loop primers from the assays of TaqMan MiRNA and by using reagents from the TaqMan® MIRNA Reverse Transcription Kit (Cat. No.4366596). The second stage was Quantitative real time PCR (qPCR) in which amplification of PCR products from cDNA

samples was done using the TaqMan MIRNA Assay (Cat. No.4427975) with also the TaqMan® Universal PCR Master Mix (Cat. No.4440043). The internal control used was MIRNA 39 (spike in control). Total sample Volume was 20 μL. Concentration of RNA taken was 100 ng/sample. For each sample,1.33 μL of cDNA was added to 10 μL of universal master mix, 7.67 μL of H_2O and 1 μL of TaqMan MIRNA Assay. Thermal Cycling conditions were as follows: one cycle of initial enzyme activation step takes 10 minutes at 95°C then 40 cycles for denaturation, annealing and extension. Each denaturation step took 15 seconds at 95°C and each annealing/extension step took 60 seconds at 50°C. The fold change (expression level) between a sample and a normal control was calculated via Relative quantification method ($RQ=2-\Delta\Delta CT$).

Determination of serum level of AFP(14)

This was achieved via ELISA kit supplied by NOVA, CAT. NO: In-Hu4125, Keyuan Road, DaXing Industry Zone, Beijing, China. The principle of the essay is a Sandwich-ELISA. AFP antibodies were present for pre-coating the microelisa strip plate. Afterward, wells were used for standards or samples, followed by addition and incubation of a Horseradish Peroxidase (HRP)-conjugated antibody specific for AFP to every well. Wells containing AFP and anti-HRP conjugated AFP became blue followed by yellow coloration after adding the stop solution. Spectrophotometric measurement of the optical density (OD) was determined at 450 nm wavelength.

Receiver-Operating Characteristic (ROC) curve: (15, 16)

ROC curve analysis was applied to assess the diagnostic performance of serum AFP and MIR-210 in HCC patients versus the cirrhosis and control groups.

At various cut-off points, the true positive rate (Sensitivity) is plotted against the false positive rate (100-Specificity). Every point on this curve can be considered as a sensitivity/specificity pair according to a certain decision threshold. So the marker with the best discrimination is the one that its ROC curve passes in the upper left corner (100% sensitivity, 100% specificity). So, if the ROC curve is closer to the upper left corner, the accuracy of the biomarker will be higher.

The data statistical analysis: (17)

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp).(18) Quantitative data were expressed as range (minimum and maximum), standard deviation and median. Kruskal Wallis test was used to compare different groups for not normally distributed quantitative variables and followed by Post Hoc test (Dunn's for multiple comparisons test) for pairwise comparison. Significance of the obtained results was judged at the 5% level.

Results

In this research, we aimed at studying MIR-210 expression level and serum AFP level 6in patients with HCC (group I), liver cirrhosis without cancer (group II) and normal control group (group III), Group I was subdivided into three subgroups IA, IB, IC representing HCC stages 0/A, B, C/D respectively.

Results were as follows:

Comparison of studied parameters among different groups:

Serum MIR-210 expression level was significantly higher in group I as compared to both groups I and II ($P < 0.001$). Serum AFP was significantly higher in group I than both groups II and group III ($P < 0.001$). **(Table 1**)

Table (1): Comparison of studied parameters among different groups

	Group1 HCC (n = 60)	Group 2 cirrhosis (n = 60)	Group 3 control (n = 60)	H	p
MIR210					
Min. – Max.	0.037 – 36.333	0.329 – 14.064	0.238 – 2.579		
Median (IQR)	4.384 (0.970 – 11.860)	2.065 (1.174 – 6.087)	1.173 (0.647 – 1.377)	36.728*	<0.001*
Sig. bet. grps.		p_1=0.047*,p_2<0.001*,p_3<0.001*			
AFP					
Min. – Max.	2.0 – 20600.0	0.10 – 508.0	1.0 – 31.0	85.158*	<0.001*
Median (IQR)	23.25(8.0 – 569.5)	4.09 (2.18 – 8.62)	2.10 (1.46 – 2.85)		
Sig. bet. grps.		p_1<0.001*,p_2<0.001*,p_3<0.001*			

MIR-210: micro RNA 210, AFP: alpha-feto protein, HCC: hepatocellular carcinoma, IQR: inter quartile range

sig. bet. grps: significance between groups

h: h for kruskal wallis test, pairwise comparison bet. each 2 groups were done using post hoc test (dunn's for multiple comparisons test)

p: p value for comparing between the studied groups

p1: p value for comparing between group 1 and group 2

p2: p value for comparing between group 1 and group 3

p3: p value for comparing between group 2 and group 3

*: statistically significant at $p \leq 0.05$

Comparison of studied parameters in different subgroups (HCC patients)

Serum MIR-210 expression level was significantly higher in group 1C than both groups 1B and 1A (P <0.001). Serum AFP showed no significant differences between subgroups IA, IB and IC. (**Table 2**)

Table (2): Comparison of studied parameters in different subgroups (HCC patients):

	Group 1A (n = 20)	Group 1B (n = 20)	Group 1C (n = 20)	H	p
MIR210					
Min. – Max.	**0.037 – 0.989**	**3.376 – 20.607**	**4.130 – 36.333**	**43.311***	**<0.001***
Median (IQR)	**0.941 (0.808 – 0.970)**	**4.705 (3.718 – 11.860)**	**13.136 (8.657 – 20.400)**		
Sig. bet. grps.	**p1<0.001*,p2<0.001*,p3=0.046***				
AFP					
Min. – Max.	**3.30 – 143.0**	**3.20 – 6000.0**	**2.0 – 20600.0**	**1.536**	**0.464**
Median (IQR)	**14.45 (9.70 – 35.25)**	**20.50 (10.30 – 662.0)**	**65.30 (4.15 – 1052.5)**		

MIR-210: micro RNA 210, AFP: alpha-feto protein, BCLC: barcelona clinic liver cancer

sig. bet. grps: significance between groups

group 1a: HCC patients with BCLC stages 0/a

group 1b: HCC patients with BCLC stages b

group 1c: HCC patients with BCLC stages c/d

IQR: inter quartile range

h: h for kruskal wallis test, pairwise comparison bet. each 2 groups were done using post hoc test (dunn's for multiple comparisons test)

p: p value for comparing between the studied subgroups

p1: p value for comparing between group 1a and group 1b

p2: p value for comparing between group 1a and group 1c p3: p value for comparing between group 1b and group 1c

*: statistically significant at $p \leq 0.05$

Receiver-Operating Characteristic (ROC) curve

ROC curve analysis was applied to assess the diagnostic performance of serum AFP and MIR-210 in HCC patients versus the cirrhosis and control groups. It revealed that serum AFP at cut-off level of 4.1 ng/ml could significantly predict the occurrence of HCC with a diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 88.33%,70.83%, 60.2%, and 92.4% respectively.

While for MIR-210, sensitivity was 66.67%, specificity 82.5%, PPV 65.6% and NPV 83.2% at cut-off level of 3.21. (**Table 3**) (**Figure 1**)

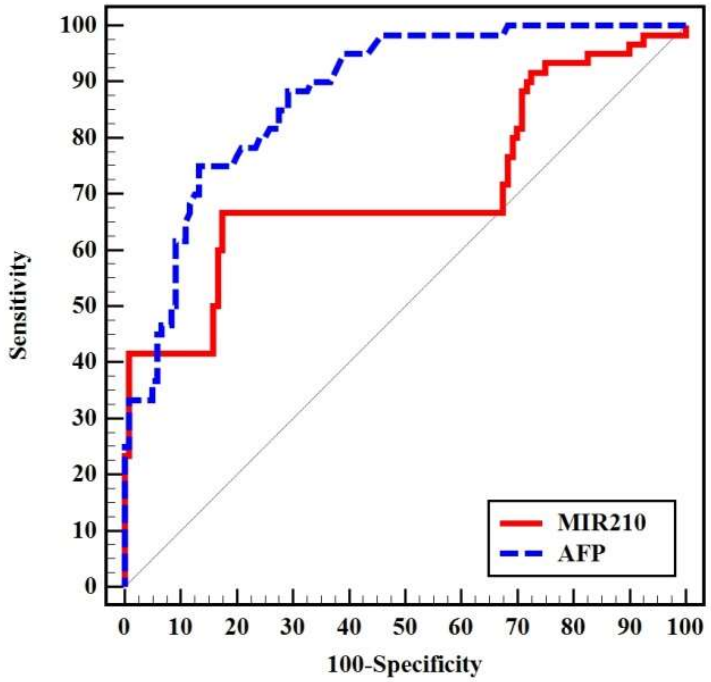

Figure (1): ROC curve for MIR210 and AFP to discriminate cancer patients (n = 60) from cirrhosis and control group (n = 120)

Table (3): Diagnostic performance for MIR210 and AFP to discriminate cancer patients (n = 60) from cirrhosis and control group (n = 120)

	AUC	p	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
MIR210	0.710	<0.001*	0.620 – 0.800	>3.21#	66.67	82.50	65.6	83.2
AFP	0.878	<0.001*	0.828 – 0.927	>4.1	88.33	70.83	60.2	92.4

AUC: Area Under a Curve

p value: Probability value

CI: Confidence Intervals

NPV: Negative predictive value

PPV: Positive predictive value

*: Statistically significant at $p \leq 0.05$

#Cut off was choose according to Youden index

Discussion

Early detection and surveillance can increase the probability of being cured from dangerous tumors such as HCC. However, even in countries with advanced medical services, there still exists the problem of underutilization of HCC surveillance.(19)

AFP is usually used as a biomarker in clinical practice to diagnose HCC but it has critical defects in the early diagnosis and prediction of cancer progression. For example, there are still a large number of HCC patients who have normal levels of AFP.(20)

This leads to poor prognosis of HCC because of late diagnosis at a non-resectable stage and inability to prevent the cancer progression at an earlier stage. Markers related to the hypoxic state accompany any cancer, MIRNAs and other molecular biomarkers have been considered promising in HCC diagnosis.(21)

A great effort has been achieved to understand the molecular mechanisms of HCC. The protein-coding genome of liver cancers has been thoroughly identified. The current work aimed at studying serum MIR-210 expression in different stages of hepatocellular carcinoma.

In the current study, MIR-210 expression level was significantly higher in HCC patients than in cirrhotic patients furthermore than that in normal individuals. In addition, MIR-210 expression was significantly higher in subgroup 1C than 1B and furthermore than 1A. This suggests that MIR-210 might play an important role not only in the tumor development but also in tumor progression.

In accordance with our study, Ji et al.,(22) revealed that increased MIR-210 expression level was correlated with poor prognosis of HCC cases. Also, Dai et al.,(11) showed that MIR-210 expression level was highly increased in the late T stages in comparison with that in the early T stages.

Hypoxia is a major characteristic feature of solid tumors to facilitate cancer growth and survival by stimulating different signaling pathways that leads to cellular angiogenesis and proliferation. Increased ROS production which leads to damage of mitochondrial DNA is induced by hypoxia.(23)

The master hypoxamir, MIRNA-210, plays a great role in many signaling pathways related to cancer development and progression, such as Ras signaling, (tumor growth factor-β) TGF-β signaling, and phosphatidylinositol 3-phosphate (PI3P) signaling.(23)

These hypoxia-related signaling pathways are extremely crucial in enhancing tumor cell survival, motility, development of angiogenesis and promoting tumor invasion and metastasis. MIR-210 also affects the signaling pathways related to stem cell pluripotency.(22, 23)

It was reported that in cancer, in vivo, MIR-210 level is correlated with a gene expression signature of hypoxia, termed hypoxia metagene.(24) Based on such data, MIR-210 expression appears to be a mirror of HIF activity in cancer,(25) thus paving the road for its use of as a marker of hypoxia in tumors.

MiRNA-210 is highly expressed in most solid tumors. Unfortunately, miRNA-210 has been found to be associated with poor clinical outcome. The role of miRNA-210 in cancer development has been thoroughly determined, and it was revealed that miRNA-210 has oncogenic features, as its levels are typically increased in tumors such as glioma, lung, liver, head and neck, colorectal and pancreatic cancers.(12)

In fact, multiple studies revealed that miRNA-210 levels might be increased by the hypoxic effect in the majority of human solid malignancies including HCC. Furthermore, miRNA-210 can enhance migration and invasion of liver cancerous cells. MiRNA-210 can be considered as a (versatile) molecule that controls cell cycle and determines many aspects of tumor cell in hypoxic conditions. It was revealed that miRNA-210 could help cancerous tumors to adapt the hypoxic stress, through suppressing the mitochondrial function, enhancing glycolysis, and stimulating angiogenesis.(26)

The diagnostic performance of the chosen biomarkers was studied using receiver operating characteristic (ROC). It was found that AFP has the highest sensitivity (88.33%) but MIR-210 has the highest specificity (82.5%). These findings indicate a relative potential of serum MIR-210 for discrimination of liver cancer from cirrhotic patients and from normal individuals.

EK Ahmed (2019) performed ROC curve analysis for distinguishing metastatic liver tumor cases from those with primary HCC and reported that MIR-210 could be confirmed as a biomarker, with an AUC of 0.67. At the cut-off value of 0.916, the maximal specificity and sensitivity were 64.28 % and 73.68%, respectively.(26)

This study has some potential limitations that could be taken into consideration in the future research. First, insufficient sample size and population traits. Second, unequal number of males and females. Third, difficulty in data collection and sampling technique. Fourth, limited resources for the study.

Conclusion

MIR-210 expression was significantly increased in serum of patients with HCC with the highest expression in the advanced cases and the lowest expression in the early cases. These results might

suggest a predictive role of MIR-210 in HCC development and progression (Risk assessment) and consequently the possibility of early treatment and prevention of HCC progression.

List of abbreviations:

HCC: Hepatocellular carcinoma
AFP: Alpha-feto protein
CT: Computed tomography
MRI: Magnetic resonance imaging
ROS: Reactive oxygen species
MIRNAs: micrornas
MIR-210: Micro RNA 210
BCLC: Barcelona clinic liver cancer
HBV: Hepatitis B virus
HCV: Hepatitis C virus
ELISA: Enzyme-linked immunosorbent assay
cDNA: complementary DNA
qPCR: Quantitative real time PCR
HIF-1α: Hypoxia-inducible factor-1α
TGF-β: Tumor growth factor-β
PI3P: Phosphatidylinositol 3-phosphate
ROC: Receiver-Operating Characteristic
AUC: Area under the curve
HRP: Horseradish Peroxidase

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval statement

This work was performed in accordance with the principles of the 1964 Declaration of Helsinki. Ethics Review Board of the Faculty of Medicine, Alexandria University has accepted the research and the ethics committee approval number was 0201432.

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