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A Study of MicroRNA-24 Expression in Aflatoxin B1 Exposed Patients with Hepatocellular Carcinoma and Cirrhosis

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Abstract

Introduction: Over 80% of hepatocellular carcinoma (HCC) sufferers occur in developing countries as a result of exposure to hepatitis B or C viruses, through formation of cirrhosis or viral integration into host DNA on the part of HBV, or due to ingestion of aflatoxin B1 (AFB1) causing DNA damage in hepatic tissue with generation of mutations, particularly in p53 tumor suppressor gene. MicroRNAs are noncoding RNAs that have an effect on oncogene and tumor suppressor gene expression, hence partaking in carcinogenesis. A connection between HCC and dysregulated expression of microRNAs has been repeatedly demonstrated, suggesting that circulating microRNAs might potentially be used as biomarkers for pre-clinical HCC detection. We aimed in this study to assess the role of micro-ribonucleic acid-24 (microRNA-24) expression in patients with cirrhosis and HCC who have experienced high levels of AFB1 exposure.

Materials and methods: Fifty HCC and 24 hepatic cirrhosis patients, in addition to 20 healthy control subjects were included in this study. Aflatoxin B1 was measured by enzyme-linked immunosorbent assay (ELISA) and microRNA-24 was detected using real-time polymerase chain reaction (real-time PCR).

Results: Both aflatoxin B1 levels and microRNA-24 expression were found to be significantly increased in all patient groups in comparison to controls, more so in the HCC than cirrhotic group (p<0.0001). A highly significant correlation was detected between levels of AFB1 and amount of microRNA-24 expressed in both patient groups relative to their control counterparts (p<0.0001). Receiver Operating Characteristic (ROC) curve performed to evaluate the ability of microRNA-24 to differentiate between HCC and cirrhosis showed that it had sensitivity of 80% and specificity of 63% at cutoff 1.3, which was highly significant (p<0.0001).

Conclusion: Increased aflatoxin B1 levels detected in patients with cirrhosis and HCC further support previous studies evaluating the level of exposure of the Egyptian population to this carcinogen and support the critical role of aflatoxin B1 in the appearance of HCC. In addition, microRNA-24 expression levels demonstrated in both cirrhosis and HCC might be valuable for use as a noninvasive diagnostic tool for diagnosis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is a potentially life-threatening affliction that constitutes over 90% of primary hepatic malignancy. This cancer is the sixth most common worldwide, accounting for a third of all cancer-related deaths [1]. Over 80% of HCC sufferers occur in developing countries most commonly as a result of exposure to hepatitis B or C viruses (HBV or HCV) or ingestion of aflatoxin B1 (AFB1) that contaminate various crops, such as corn and peanuts, in hot humid climates. These two principal risk factors of HCC are predominantly found in highly endemic regions of Africa and Asia [2,3].

Several studies have demonstrated excessively high levels of aflatoxin exposure to the Egyptian community. AFB1 is the most common aflatoxin, originating from the *Aspergillus flavus* and *Aspergillus parasiticus* mold species [4]. Exaggerated concentrations of AFB1 have been detected in a number of cultivated food products, including wheat, corn, peanuts, fava beans, and both brown and white rice, from various urban and rural districts in Egypt [5], while AFB1 was found in serum and urine of Egyptian infants with protein-energy malnutrition [6].

From a pathogenic perspective, exposure to AFB1 promotes DNA damage with generation of characteristic mutations in hepatic tissue, particularly with regard to the p53 tumor suppressor gene, a mutation found in over 50% of AFB1-exposed HCC patients [7]. In addition, chronic infections with hepatitis B or C viruses cause liver damage with development of cirrhosis which, when coupled with the supplemental ability of hepatitis B viral DNA to integrate into the host genome, all contribute to an increased risk of development of HCC [8].

Detection of HCC while in its initial stages is of paramount importance, as evidenced by a 5-year overall survival (OS) rate exceeding 50% when the disease is detected early compared to less than 10% with late HCC detection associated with poor prognosis as result of metastasis and recurrence [9]. Therefore, therapeutic advances regarding HCC metastasis and recurrence are dependent on better comprehension of the elaborate molecular machinery determining disease aggression and evolution, as well as searching for important diagnostic markers and therapeutic targets [10].

Several studies have demonstrated a connection between HCC and the abnormal expression of microRNAs [11]. MicroRNAs are short, endogenous, non-coding RNAs that coordinate the translation of protein-coding genes by either abolishing translation of target mRNAs or enhancing degradation of mRNA [12], thus regulating gene expression in a quick sensitive manner [13]. MicroRNA disorganization has been implicated in a multitude of disorders, including cancer, through modification of natural cell development and differentiation [14].

The high stability of microRNAs in circulation, added to the fact that microRNA expression patterns appear to be tissue-specific, sets the stage for detection of circulation microRNAs to be potentially used as ideal biomarkers for pre-clinical HCC [15]. Several studies have confirmed the ability of serum microRNAs to recognize early-stage HCC caused by infections with hepatitis B and C viruses [16,17]. However, very limited data exist correlating serum microRNA expression with HCC caused by AFB1 exposure. Therefore, analysis of microRNAs in circulation of patients with cirrhosis and HCC exposed to AFB1 may offer a clinically applicable non-invasive diagnostic means for detection of curable stages of this malignancy. Amid more than 2000 human microRNAs defined to date, microRNA-24 has been found to modulate the development of cancer in a variety of malignancies, including HCC [18,19].

Therefore, we aimed in this study to assess the role of circulating microRNA-24 expression in patients with cirrhosis and those with HCC who have experienced high levels of AFB1 exposure, in addition to studying the role of AFB1 exposure as an associated risk factor for development of HCC.

Material and Methods

Subjects

This case-control study was conducted on 74 patients with hepatic cirrhosis due to hepatitis C virus (HCV) presented to Mansoura University Hospital during the period from January 2016 until March 2017. Of these, 50 patients had hepatocellular carcinoma and 24 patients were HCC free. In addition, 20 healthy subjects were recruited as control group.

Patients included in the study were those over 18 years of age who were positive for HCV antibodies with cirrhosis or HCC in absence of other known malignancies. Due to its established role as a direct participant in the hepatocarcinogenic process and so as to avoid confounding results, patients with HBV were excluded, as were patients with HIV. All patients were subjected to thorough clinical, laboratory, and radiological assessment. Cirrhotic patients were diagnosed based on imaging (ultrasound or CT) findings and characteristic liver function abnormalities, then classified according to Child-Pugh score.

In case of presence of elevated α-fetoprotein >400 ng/ml, diagnosis of HCC was confirmed with one positive imaging study, such magnetic resonance imaging (MRI) and/or computerized tomography (CT), while cases with normal α-fetoprotein level required two positive imaging studies [20].

Blood sampling

Ten milliliters of blood were taken from each participant, which were then divided into three aliquots. One aliquot contained blood over EDTA from which plasma was separated for microRNA-24 extraction, while another aliquot was a plain aliquot for sera separation for determination of aflatoxin B1 by immunoassay. Sera that was separated from the third aliquot was used for laboratory study of complete liver functions by autoanalyzer (Dialab 450 system), determination of circulatory anti-HCV by Elecys system (Roche-diagnostic) and alpha fetoprotein (AFP) measurement by enzyme linked immunosorbant assay (ELISA - DRG International Inc., USA.).

Measurement of ALT, AST, total bilirubin and direct bilirubin

By Dialab system and kits.

### Measurement of ALT (GPT) (Glutamate Pyruvate Transaminase)

In vitro detection of ALT (GPT) in serum requires kinetic quantification on photometric systems using a diagnostic reagent. The modified formulation for assessment of GPT approved by the International Federation of Clinical Chemistry (IFCC) is as follows:

L-Alanine+2-Oxoglutarate <GPT> Pyruvate+L-Glutamate Pyruvate +NADH + H^+ <LDH> L-Lactate+NAD^+

Oxidation of NADH to NAD^+ results in an absorbance reduction at 340 nm, a directly proportionate measure of GPT activity in the sample. This method cited by the IFCC incorporates pyridoxal phosphate (P-5-P), which acts as a coenzyme in transfer of AA, thereby stabilizing transaminase activity. Hence, values that may be falsely low due to deficient endogenous P-5-P sample content, as in patients with liver disease or myocardial infarction, can be avoided by addition P-5-P to the studied sample [21].

Measurement of AST (GOT) (Glutamate Oxaloacetate Transaminase)

In vitro detection of AST (GOT) in serum requires kinetic quantification on photometric systems using a diagnostic reagent. The modified formulation for assessment of GPT approved by the International Federation of Clinical Chemistry (IFCC) is as follows:

L-Aspartate+2-Oxoglutarate GOT Oxaloacetate+L Glutamate

Oxaloacetate+NADH+H^+ <MDH L-Malate+NAD^+
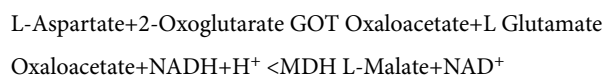
Oxidation of NADH to NAD+ results in an absorbance reduction at 340 nm, a directly proportional measure of GOT activity in the sample. This method cited by the IFCC incorporates pyridoxal-5'-phosphate (P-5-P), which acts as a coenzyme in transfer of AA, thereby stabilizing transaminase activity. Hence, values that may be falsely low due to deficient endogenous P-5-P sample content, as in patients with liver disease or myocardial infarction, can be avoided by addition P-5-P to the studied sample [21].

Measurement of total bilirubin and direct bilirubin

In vitro detection of direct and total bilirubin in serum requires kinetic quantification on photometric systems using a diagnostic reagent.

Test Principle

The heme fraction of hemoglobin discharged by old or dying red blood cells forms the origin of bilirubin by conversion in the liver to either bilirubin monoglucuronide or diglucuronide. Because free bilirubin in insoluble in aqueous solution, it requires dissolution by solvents such as alcohols to become reactive, this providing the calculation for total bilirubin. Bilirubin monoglucuronide or diglucuronide are soluble in aqueous solution, thereby allowing for measurement of what in this form is referred to as direct bilirubin. Dimethylsulfoxide (DMSO) and ethylene glycol are utilized as solvents in the kit for assessment of total bilirubin. These allow bilirubin to readily react with diazotized sulfanilic acid with production of a deeply colored diazo dye, color intensity of which is proportional the amount of direct and total bilirubin, respectively.

AFP (ELISA-DRG International Inc., USA.)

The DRG AFP ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. The microtiter wells are coated with a monoclonal (mouse) antibody directed towards a unique antigenic site on an AFP molecule. An aliquot of sample containing endogenous AFP is incubated in the coated well with enzyme conjugate, which is an anti-AFP antibody conjugated with horseradish peroxidase. After incubation the unbound conjugate is washed off. The amount of bound peroxidase is proportional to the concentration of AFP in the sample. Having added the substrate solution, the intensity of color developed is proportional to the concentration of AFP in the sample. The absorbance was read by ELISA humareader (Human) microplate reader.

AFB1 measurement by ELISA

The aflatoxin B1 (AFB1) level in serum samples was determined by (Ridascreen- R-Biopharm AG, Demstadt, Germany), an ELISA kit based on competitive enzyme immune assay for the quantitative analysis of aflatoxin B1. Preparation of serum samples required pre-dilution with a dilution buffer (8.75 ml sample buffer of Ridascreen and 1.25 ml methanol) [22].

The procedures were implemented as per the manufacturer's manual. Positive controls AFB1 were included in the kit, these containing 4000, 2000, 1000, 500, 250 or 0 ng/kgm in methanol/water (10/90) to allow for standard curve plotting for interpretation of results. The kit is dependent on the principle of competitive enzyme immunoassay. The microplate is coated with anti-aflatoxin antibodies, followed by addition of the serum in addition to second aflatoxin antibodies and conjugate enzymes. Aflatoxin antibody binding sites are competed for by free aflatoxin and aflatoxin enzyme conjugate. Simultaneously, the anti-aflatoxin antibodies are also bound by the immobilized capture antibodies. Any unbound enzyme conjugate is then removed by washing, after which chromogen substrate was added to the wells and transformed into a blue product by bound enzyme conjugate. A colour change from blue to yellow occurs when stop solution is added, allowing for photometric measurement to be performed at 450 nm, with absorbance being inversely proportional to sample aflatoxin concentration. Results were expressed as ppb AFB1 per blood serum.

MicroRNA-24 Assessment by Real-Time PCR

MicroRNA isolation

MicroRNA was prepared from 200 μl serum samples by the use the microRNA extraction kit (Qiagen) according to the manufacturer's instructions. All isolated microRNAs were aliquoted and stored in a freezer at −80°C until use.

cDNA synthesis

Reverse transcription (RT) was carried out in a 20 μl solution that contained 0.2 μl 200 U/μl reverse transcriptase (Qiagen), 0.2 μl, 40 U/μl ribonuclease inhibitor, 0.8 μl, 10 mmol/ml dNTP mix, 1.2 μl, 10 mmol/ml stem-loop RT primer, 4 μl RT buffer, 11.6 μl RNAase-free water and 2 μl microRNA template. After gentle mixing, the reaction mixtures were incubated at 25°C for 5 min, 40°C for 60 min and then 70°C for 15 min. The final cDNA products were stored at −20°C until use. The reverse transcription primer was miR-24 [GenBank: AF480527.1], 5′-GTCGTATCCAGTGCGTGTCGGGAGTCGGCAATTGCACTGATACGACCTGTTCCT-3′. According to the method of Fang et al. 2015 one primer is used for cDNA synthesis.

Quantitative real-time PCR

Real-time PCR was carried out following the manufacturer's protocol of SYBR Premix Ex Taq TM II reagents with 2 μl cDNA template (Qiagen). All real-time PCR reactions were carried out on an ABI7500 (ABI) machine. Reaction conditions were 95°C for 30s, followed by 40 cycles of 95°C for 5s and 60°C for 34s. The primers were: miR-24, 5′-GCAATGTGGCTCAGTTCAG-3′ (forward), 5′-CAGTGCGTGTCGTGGAGT-3′ (reverse) and the probe sequence was miR-24, probe sequence 5′-CUGUUCCUGCUGAACUGAGCCA-3′. The system used for real time PCR was Applied Biosystem StepOne TM Real-Time PCR system [23].

Statistical analysis

All statistical analyses were performed using SPSS 16.0 (Chicago, IL) and a significance threshold of $P<0.05$ was used. Continuous data were expressed as mean ± SD and compared between groups using Bonferroni test. Categorical data were analyzed using a two-sided Chi-squared test or Fisher's exact test, when appropriately indicated. The receiver operating characteristic (ROC) curve was done to differentiate between groups.

Ethical approval

Informed consent was taken from each patient. The research protocol was approved by the Ethical Committee of Faculty of Medicine, Mansoura University (proposal code: R.18.02.53).

Results

Different grading of cirrhosis and HCC patients is demonstrated in Table 1. All patients were arranged according to Child-Pugh classification [24]. A large number of patients included in this study had decompensated liver disease and thus were given a Child class of C

(14 cirrhotic and 20 HCC patients). Over half of HCC patients (30 patients; 60%) suffered from a solitary HCC lesion, with the vast majority of patients (72%) being staged as A and B (20 patients (40%) and 16 patients (32%), respectfully) by the Barcelona Clinic Liver Cancer (BCLC) staging system [25].

Grading		Cirrhosis (n=24)	HCC (n=50)	p value
Child-Pugh classification	A	5	15	
	B	5	15	0.33
	C	14	20	
Number of masses	Single: n (%)		30 (60)	-
	Multiple: n (%)		20 (40)	
BCLC	A: n (%)		20 (40)	
	B: n (%)		16 (32)	-
	C: n (%)		12 (24)	
	D: n (%)		2 (4)	

*The p value indicates the statistical significance for the differences between cirrhosis and HCC groups.

The mean difference is significant at a level of 0.05.

BCLC: Barcelona Clinic Liver Cancer staging system.

Table 1: Different grading of the cirrhosis and HCC groups.

Aflatoxin B1 levels measured in study subjects are shown in Table 2, and were shown to be significantly elevated in all patient groups of cirrhosis (3.7 ± 1.9) and HCC (5.4 ± 2.1) when compared to the control group (1.2 ± 0.4). Similarly, the increased AFB1 level was also found to be statistically significant in the HCC group in comparison to the group with cirrhosis (p<0.0001).

Aflatoxin B1 (ppb)	Control (n=20)	Cirrhosis (n=24)	HCC (n=50)
Mean ± SD	1.2 ± 0.4	3.7 ± 1.9	5.4 ± 2.1
Minimum	0.5	1.5	2.5
Maximum	1.8	8	9
Bonferroni 1		<0.001*	<0.001*
Bonferroni 2	<0.001*	<0.001*	
Bonferroni 3	<0.001*		<0.001*
ANOVA	38.3 (p<0.0001)*		

Bonferroni 1: Comparison of control versus HCC and cirrhosis groups.
Bonferroni 2: Comparison of HCC versus control and cirrhosis groups.
Bonferroni 3: Comparison of cirrhosis versus control and HCC groups.
The mean difference is significant at a level of 0.05.
ppb: Part per billion.

Table 2: Statistical analysis of aflatoxin B1 levels in the different studied groups.

Evaluation of microRNA-24 expression in the different studied groups is demonstrated in Table 3. MicroRNA-24 was found to be

significantly higher in both HCC (2.6 ± 1.3) and cirrhotic (1.7 ± 1.3) patient groups when compared with controls (0.6 ± 0.3), with this elevation being more prominent in the HCC group than in cirrhotic patients, and in the cirrhotic group more than control individuals (p<0.0001).

MicroRNA-24	Control (n=20)	Cirrhosis (n=24)	HCC (n=50)
Mean±SD	0.6 ± 0.3	1.7 ± 1.3	2.6 ± 1.3
Minimum	0.5	1.5	2.5
Maximum.	1.8	8	9
Bonferroni 1		<0.01*	<0.001*
Bonferroni 2	<0.001*	<0.01*	
Bonferroni 3	<0.01*		<0.01*
ANOVA	20.9 (p<0.0001)*		

Bonferroni 1: Comparison of control versus HCC and cirrhosis groups.
Bonferroni 2: Comparison of HCC versus control and cirrhosis groups.
Bonferroni 3: Comparison of cirrhosis versus control and HCC groups.
The mean difference is significant at a level of 0.05.

Table 3: Statistical analysis of microRNA-24 expression in the different studied groups.

In Table 4, although AFP and aflatoxin B1 levels were increased in Child A and C when compared to Child B cirrhotic patients, microRNA-24 expression was found to be increased in Child A more than Child B and C. However, no statistical differences were found between AFP, microRNA-24 and aflatoxin B1 and Child classes A, B and C in this group of patients with cirrhosis.

Variables	Child-Pugh classification		
	A n=5 (20.8%)	B n=5 (20.8%)	C n=14 (58.3%)
AFP	14.8 ± 10.6	6.2 ± 3.5	10.9 ± 11.9
ANOVA	0.838, p=0.446		
Bonferroni1	P=0.63		
Bonferroni2	P=1.000		
Bonferroni3	P= 1.000		
MicroRNA-24	1.81 ± 1.54	1.60 ± 1.47	1.65 ± 1.41
ANOVA	0.02, p=0.974		
Bonferroni1	P=1.000		
Bonferroni2	P=1.000		
Bonferroni3	P=1.000		
Aflatoxin B1	3.88 ± 2.1	3.48 ± 1.3	3.72 ± 2.1
ANOVA	0.05, p=0.951		
Bonferroni1	P=1.000		
Bonferroni2	P=1.000		

Bonferroni3	P=1.000

The p value indicates the statistical significance for the differences among groups.
AFP: Alfa-fetoprotein, n: number.
Bonferroni 1: Comparison of Child A versus Child B.
Bonferroni 2: Comparison of Child A versus Child C.
Bonferroni 3: Comparison of Child B versus Child C.
ANOVA: Analysis of Variance, n: number.

Table 4: Statistical differences of AFP, microRNA-24 and aflatoxin B1 between Child (A, B & C) in cirrhosis group.

In hepatocellular patients, AFP level was found to be increased in Child class C more than classes A and B. Conversely, microRNA-24 expression demonstrated increased levels in Child A and C patients more than in patients classified as Child B, with aflatoxin B1 levels being increased in Child A patients when compared with Child B and C patients. However, once again there were no statistical differences between AFP, microRNA-24 and aflatoxin B1 and Child A, B and C the hepatocellular group of patients as shown in Table 5.

Variables	Child-Pugh classification		
	A n=15 (30%)	B n=15 (30%)	C n=20 (40%)
	-0.3	-0.3	-0.4
AFP	420.2 ± 818.1	533.1 ± 818.8	591.3 ± 845.6
ANOVA	0.184, p=0.833		
Bonferroni1	P=1.000		
Bonferroni2	P=1.000		
Bonferroni3	P=1.000		
MicroRNA-24	2.73 ± 1.3	2.33 ± 1.4	2.72 ± 1.3
ANOVA	0.464, p= 0.631		
Bonferroni1	P=1.000		
Bonferroni2	P=1.000		
Bonferroni3	P=1.000		
Aflatoxin B1	5.82 ± 2.2	5.06 ± 1.9	5.44 ± 2.1
ANOVA	0.474, p= 0.625		
Bonferroni1	P=1.000		
Bonferroni2	P=1.000		
Bonferroni3	P=1.000		

The p value indicates the statistical significance for the differences among groups.
AFP: Alfa-fetoprotein, n: number.
Bonferroni 1: Comparison of Child A versus Child B.
Bonferroni 2: Comparison of Child A versus Child C.
Bonferroni 3: Comparison of Child B versus Child C.
ANOVA: Analysis of Variance, n: number.
HCC: Hepatocellular carcinoma.

Table 5: Statistical differences of AFP, microRNA-24 and aflatoxin B1 between Child (A, B & C) in HCC group.

Pearson correlation between AFP and microRNA-24 in cirrhosis and HCC groups (Table 6) showed that no significant correlation could be found between levels of AFP and microRNA-24 expression levels in the studied groups of patients with either cirrhosis or HCC, respectively.

Groups	Pearson correlation (r)	p value
Cirrhosis group AFP MicroRNA-24	0.046	0.830
HCC group AFP MicroRNA-24	0.111	0.442

The p value indicates the statistical significance for the differences among groups
AFP: Alfa-fetoprotein, n: number
HCC: Hepatocellular carcinoma

Table 6: Pearson correlation between AFP and microRNA-24 in cirrhosis and HCC groups.

Correlation analysis between aflatoxin B1 and microRNA-24 expression in different studied groups showed no correlation between these parameters in the control group of subjects (p<0.755) (Figure 1A). However, highly significant correlation was found between the levels of aflatoxin B1 detected in cirrhotic patients and the amount of microRNA-24 expressed in these patients (r=0.933; p<0.0001) (Figure 1B), as was detected in HCC patients (r=0.868; p<0.0001) (Figure 1C).
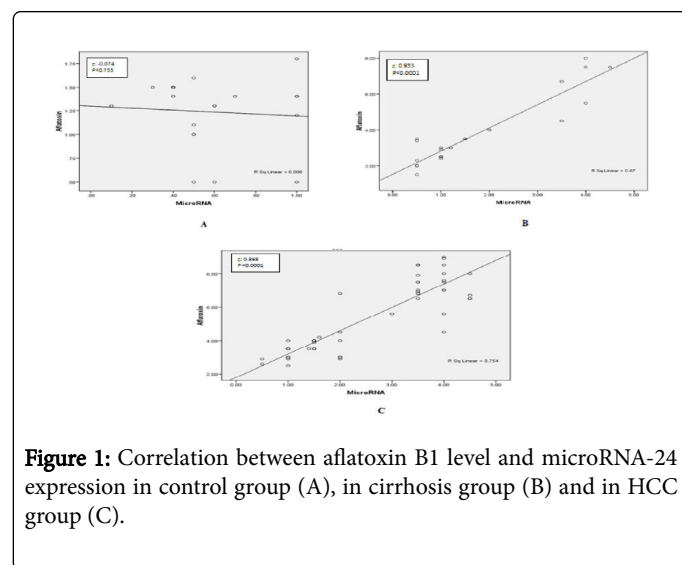

Figure 1: Correlation between aflatoxin B1 level and microRNA-24 expression in control group (A), in cirrhosis group (B) and in HCC group (C).

ROC curve analysis for microRNA-24 expression demonstrated that at a cutoff of 0.85, microRNA-24 had sensitivity of 80% and specificity of 75% in differentiating cirrhosis from normal liver of control group of subjects (Figure 2), and 98% sensitivity and 85% specificity in delineating HCC from control liver (Figure 3), both findings that were highly significant (p<0.0001). Furthermore, when differentiating between patients with HCC and those with cirrhosis, microRNA-24 demonstrated sensitivity of 80% and specificity of 63% at a cutoff of 1.3, another highly significant finding (p<0.0001) (Figure 4).
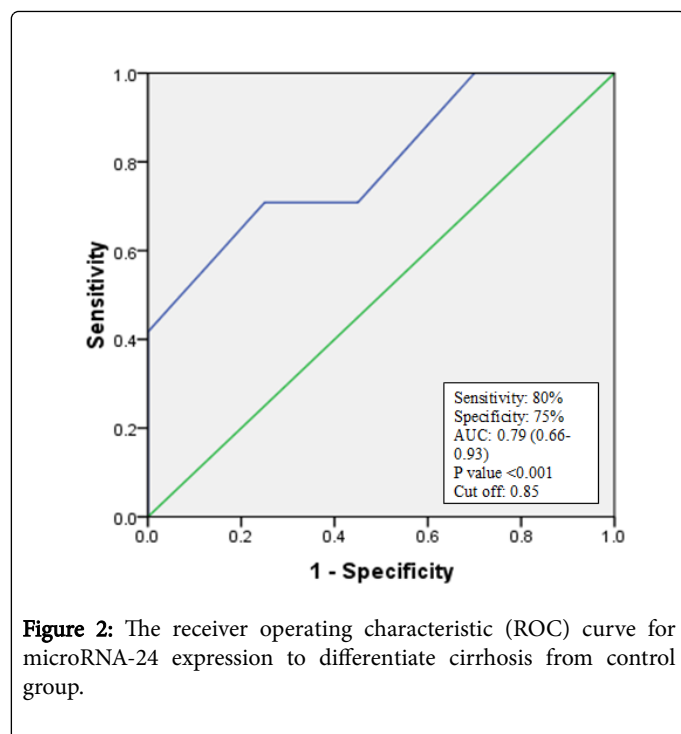
Figure 2: The receiver operating characteristic (ROC) curve for microRNA-24 expression to differentiate cirrhosis from control group.

Discussion

Ingestion of aflatoxins and exposure to hepatitis B or C viruses has been widely established as cardinal risk factors for hepatocarcinogenesis. Aflatoxin B1 (AFB1) is a mycotoxin that originates from some mold species such as *Aspergillus parasiticus* and *Aspergillus flavus* that commonly contaminate various harvested products when stored in moist environments. Ingestion of this toxin is followed by its metabolism by cytochrome P450 into aflatoxin B1-exo-8,9 epoxide (AFBO), a toxic metabolite capable of binding to DNA and causing genomic DNA damage, especially in the p53 suppressor gene, with initiation of hepatocellular carcinoma (HCC) development [2]. In addition, the end stage of viral hepatitis caused by exposure to HBV or HCV is ultimately liver damage by cirrhosis which, in addition to integration into host genome by hepatitis B viral DNA, collectively contributes to an increased risk of development of HCC [8].

The association of microRNAs with hepatocellular carcinoma has been a subject of intense analysis in recent years. By organizing gene expression, these small noncoding RNAs are capable of influencing a variety of different cell functions including cell differentiation and proliferation, metabolism, and cell apoptosis [26]. In addition, their effect on expression of oncogenes and tumor suppressor genes has established their pivotal role in pathogenesis of numerous cancers, including hepatocarcinogenesis [27,28]. The exceptional stability and tissue-specific expression patterns of serum microRNAs have strengthened their role as potential contenders for noninvasive cancer testing [29]. On this foundation, serum microRNAs have been proposed to be of potential diagnostic value for HCC, especially that related to high AFB1 exposure. Worthy of mention is the possible involvement of miRNA-24 in carcinogenesis [30]. Several studies have demonstrated persistent dysregulation of microRNA-24 in patients with hepatocellular carcinoma [30,31]. Accordingly, the current case-control study aimed to assess and analyze the diagnostic role of circulating microRNA-24 expression in patients with HCC in

comparison to patients with cirrhosis in areas of high AFB1 endemicity such as Egypt as well as to study the role of AFB1 exposure as an associated risk factor for development of HCC.
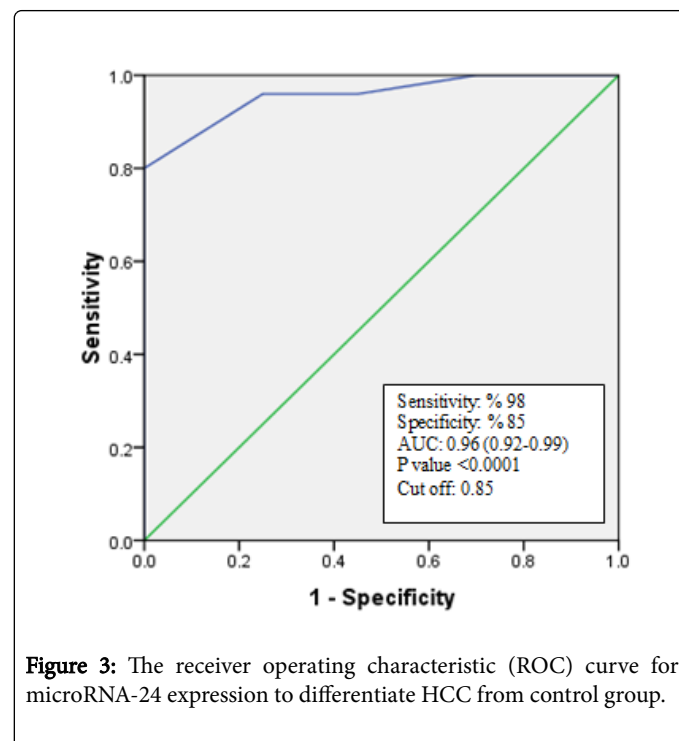

Figure 3: The receiver operating characteristic (ROC) curve for microRNA-24 expression to differentiate HCC from control group.
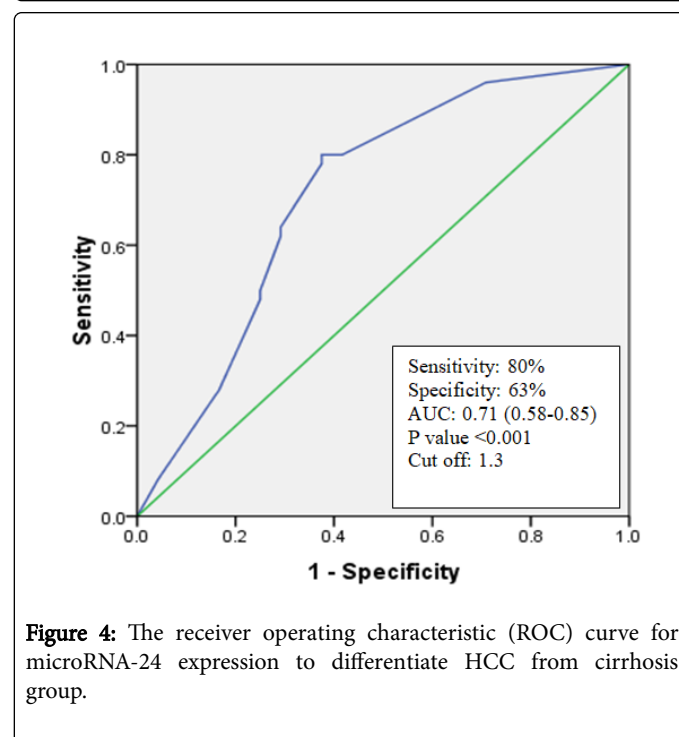

Figure 4: The receiver operating characteristic (ROC) curve for microRNA-24 expression to differentiate HCC from cirrhosis group.

Aflatoxin B1 levels evaluated in 50 patients with HCC and 24 patients with hepatic cirrhosis included in this study was shown to be significantly elevated in all patient groups when compared to the control group of subjects. In addition, the increased AFB1 level was also found to be statistically significant in the HCC group in

comparison to the group with cirrhosis. In similarity to the present study, Sharaf-Eldin et al. showed that serum aflatoxin levels were significantly increased to a greater extent in HCC patients than cirrhotic patients, and in the latter patients than in controls, possibly ascribed to the accumulative ramifications of prolonged exposure to aflatoxin with advanced age [32]. These results corroborate analogous studies conducted in Egypt demonstrating higher aflatoxin B1 levels in cases of HCC, especially if they were male farmers from rural areas afflicted with chronic HCV and presenting with cirrhosis as well as multifocal HCC lesions. These results support the critical role of aflatoxin B1 in the appearance of HCC [33].

On a similar scale, evaluation of microRNA-24 expression in the different studied groups showed that microRNA-24 was found to be significantly higher in both HCC and cirrhotic patient groups when compared with controls, with this elevation being more prominent in the HCC group than in cirrhotic patients, and in the cirrhotic group more than control individuals. Frequent dysregulation of microRNA-24 expression in liver cancer has been previously demonstrated [30,31]. In addition, various studies have exposed different expression patterns of serum microRNAs in HCCs when compared to non-HCCs [29], highlighting the ability of serum microRNAs to distinguish HCC cases from controls [9,16,34]. Taken together, results of these studies propose that serum microRNAs might be of relevance as noninvasive biomarkers for the diagnosis of HCC.

Therefore, in an attempt to determine the efficacy of microRNA-24 expression in discriminating between HCC, cirrhosis, and control subjects, ROC curve was done for all studied groups. At a cut off of 0.85, microRNA-24 was found to adequately differentiate cirrhosis from controls with sensitivity of 80% and specificity of 75%, while an even higher sensitivity of 98% and specificity of 85% was demonstrated in discerning HCC from control individuals, both findings being highly significant. Moreover, microRNA-24 was found to differentiate HCC from cirrhosis at a cut off of 1.3 with sensitivity of 80% and specificity of 63%, also a highly significant finding. Our data indicate that the overexpression of microRNA-24 detected in both our study groups of cirrhosis and HCC might be of use as a noninvasive biomarker delineating cirrhosis from HCC.

Correlation between aflatoxin B1 levels and serum microRNA-24 expression in the current study was insignificant in the control group of study subjects, while both patient groups showed highly significant correlation between these studied variables, more so in HCC patients than in cirrhotic patients. A study by Liu et al. showed that increased expression of microRNA-24 could advance AFB1-DNA synthesis, possibly as a result of their targeted effect on detoxification enzyme genes [35] resulting in diminished detoxification capacity with consequent aggregation of AFB1-DNA [10]. Furthermore, other studies showed that low microRNA expression of DNA repair would limit the scope of DNA repair thus increasing the extent of DNA damage and risk of HCC [15,36]. These observations contribute to a unique understanding of the mechanism behind AFB1-induced HCC [10].

In conclusion, our results show that increased aflatoxin B1 levels detected in patients with cirrhosis and HCC further support previous studies evaluating the level of exposure of the Egyptian population to this carcinogen and support the critical role of aflatoxin B1 in the appearance of HCC. In addition, microRNA-24 expression levels demonstrated in both cirrhosis and HCC might be valuable for use as a noninvasive diagnostic tool for diagnosis of HCC. However, further studies into the fundamental role of microRNAs and their dysregulated expression patterns involved in hepatic carcinogenesis will afford valuable insights into the intricate molecular pathogenesis of HCC with possible facilitation of novel therapies for HCC patients, particularly those from areas of high AFB1 exposure.

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