Predictive Biomarkers of Hepatocellular Carcinoma in Sudanese Patients with Liver Cirrhosis

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Abstract: _Background: Liver cirrhosis (LC) is a prevalent condition in Sudan and the main predisposing factor for hepatocellular carcinoma (HCC). This study assesses the effectiveness of the use of α-fetoprotein (AFP), ultrasound (US), AFPmRNA in addition liver enzymes as early predictors for hepatocellular carcinoma (HCC) in Sudanese patients with liver cirrhosis (LC). Methods: A Hundred patients with LC, 25 patients with confirmed HCC and 50 healthy controls were included in the study. Serum AFP, AFPmRNA and liver enzymes were determined annually in patients with liver cirrhosis in addition to abdominal U/S for 3 years. Serum AFP was determined using enzyme immunoassay (EIA) in patients and controls. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used for detection of AFPmRNA. Results: During follow up hepatic nodules were identified in three liver cirrhotic patients (3%). AFP detection rate was higher (64%) at a low cut-off point (20ng/dl). the AFPmRNA had a very low sensitivity (24%) in HCC group. Only 3 patients developed HCC additionally to LC after follow up and all had positive AFPmRNA and AFP. Conclusions: In conclusion the combination of U/S, AFP and AFPmRNA would be the most effective tool for HCC screening in Sudan. However, AFPmRNA alone had the lowest detection rate and is therefore not recommended for screening._

Keywords: hepatocellular carcinoma, α-fetoprotein, AFPmRNA, ultrasound, liver cirrhosis, Sudan

1. Introduction

Hepatocellular carcinoma (HCC) occurs mostly on top of liver cirrhosis and chronic liver inflammatory conditions (1, 2). The geographic distribution of this cancer depends on the distribution of its predisposing factors like hepatitis B virus (HBV) and hepatitis C virus (HCV). These factors were reported to cause 57% and 78% of cirrhosis and HCC cases respectively worldwide (3).

HBV and aflatoxin B1 are the main causes of liver cirrhosis and HCC in Africa (4, 5). Alcohol consumption and non-alcoholic steato-hepatitis are contributing factors in developed countries (2). Other factors such as diabetes mellitus are more common in modern life and constitute risk factors for HCC (6). In Sudan HBV and aflatoxin in peanut butter are major risk factors for development of HCC as 80% of HCC cases could be caused by these two factors (7).

Early detection of HCC is important as it can improve the survival of patients (8). Currently the recommended screening strategy includes measurement of serum α-fetoprotein (AFP) levels with abdominal ultrasound for the detection of HCC at an earlier stage (9). This strategy can increase the detection of HCC nodules three-fold and can decrease the number of deaths (10).

The extraction and detection of AFPmRNA from peripheral blood mononuclear cells by RT-PCR has been studied as molecular marker which may improve the effectiveness in screening of HCC patients for early metastasis (11).

The objectives of this study are to evaluate the use of AFP and AFPmRNA as early predictors of HCC in Sudanese patients with liver cirrhosis.

2. Material and Methods

This is a prospective case-control study carried out in National center for gastroenterology (Ibn sina hospital) in Khartoum, Sudan. The study population consisted of 100 patients with liver cirrhosis, 25 patients with hepatocellular carcinoma and 50 healthy controls. Both patients and controls were studied after obtaining informed consent. The patients with liver cirrhosis were > 20 years according to recommendations of the American association for the study of liver diseases (AASLD)(12) and were followed up for 3 years. To be enrolled in liver cirrhosis group the patients were diagnosed on clinical findings, laboratory and radiological testing and were either being Child's grade A or B (patients with child's grade C were excluded). Hepatitis B and C viruses were diagnosed by testing patients' sera for hepatitis B surface antigen and hepatitis C antibody. The follow up included clinical examination and yearly measurement of liver enzymes, alpha-fetoprotein, ultrasound scanning for detection of early HCC nodules and AFP mRNA. All patients in the hepatocellular carcinoma group were developing carcinoma on top of liver cirrhosis, and all

were diagnosed according to AASLD (12).Liver enzymes (aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT) were measured following routine spectrophotometric methods. Serum Alpha fetoprotein (AFP) was measured using a one-step sandwich EIA. AFP mRNA was detected using heparinized whole blood from which plasma fraction was removed. The cellular fraction was enriched for mononuclear cells or possible tumor cells according to the method described by Komeda (13). RNA was extracted by Qiagen-RNeasy midi kit. Nested RT-PCR was conducted and samples were analyzed by electrophoresis on a 15% polyacrylamide gel and stained with ethidium bromide for the specific bands. The design of external and inner pairs of primers were taken from previous research as follows (14).
EX-sense 5'-ACTGAATCCACAACACTGCATAG-3'
EX-antisense 5'-TGCAGTCAATGCATCTTCACCA-3'
IN-sense 5'-TGGAATAGCTTCCATATTGGATTC-3'
IN-antisense 5'-AAGTGGCTCTTGAACAAACTGG-3'

According to the design of primer pairs, the PCR products of 174 and 101 base pairs were amplified from AFP cDNA by external and internal primer pairs, respectively. The locations of the primer pairs were as follows:EX-sense in exon 1 (AFP mRNA nucleotides 90-112), EX-antisense in exon 2 (AFP mRNA nucleotides 263-241), IN-sense over exon 1 and exon 2 (AFP mRNA nucleotides 122-145), IN-antisense in exon 3 (AFPmRNA nucleotides 222-200).

3. Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) software SPSS Inc., Chicago, IL, USA, version 13.0. The Data were tested statistically for normal distribution by (Kolmogorov-Smirnov & Shapiro-Wilk) tests and were expressed as mean ± standard deviation (SD) if normally distributed or median (25th- 75th quartile) if it is abnormally distributed. Comparing between groups were assessed by Student's t-test or the Chi-square test for numerical and categorical data, respectively. Abnormally distributed numerical variables were compared by Mann-Whitney U test. P<0.05 was considered statistically significant.

4. Results

The general characteristics of the patients, 100 with liver cirrhosis, 25 with hepatocellular carcinoma and the 50 controls, are shown in table 1. Most of patients with liver cirrhosis were males (78%). The main cause of liver cirrhosis in this group was hepatitis B viral infection (42%) followed by alcohol consumption (28%) and HCV (6%), **Table 1**.
Serum AFP, AFPmRNA and liver function test (ALT, AST, GGT, ALP, serum AFP)

Patients with hepato-cellular carcinoma were divided to two groups according to AFPmRNA status, positive or negative. AFPmRNA was not detected either in patients with liver cirrhosis nor normal control. In the hepatocellular carcinoma group, the middle quartiles (25th – 75th) of serum AFP was significantly higher in AFPmRNA positive compared to AFPmRNA negative group [1000.0 (648.3-1052) vs 36.2 (4.0-122.8) ng/ml, *P*= <0.001], table 2. Although serum

ALT, AST, GGT, ALP and albumin were lower in patients with positive AFPmRNA compared to the negative group, the difference is not statistically significant.

Among patients with liver cirrhosis, significant raised levels of ALP were observed in patients with liver cirrhosis compared to normal control [31.5 (3.8) vs 7.52 (4.6) IU/, *P*= 0.01]. There was a significant decrease during follow up of patients with LC in AST (*P*<0.001), ALT (*P*=0.004), and GGT (*P*= 0.002). However, ALP enzyme levels did not change significantly with time (P=0.210), (**Table 2**).

Most of Patients with hepatocellular carcinoma on top of liver cirrhosis were males (68%), mainly in age group (>60yrs) 48%; the predisposing factors were HBV (44%) followed by alcohol consumption (20%), then HCV (16%).

An AFP cut off level ≥20 ng/dl was chosen in line with other studies (15); the detection rate by AFP for tumor among HCC group is 64% (16/25). The detection rate of AFPmRNA is only 24% in the HCC group. 66.7% of patients with HCC in whom AFPmRNA was detected were positive for HBV.

AFPmRNA were detected in all three patients (3/100) who developed HCC additionally to liver cirrhosis during the follow-up period and they also have AFP > 20 ng/dl. During the follow-up period, three patients died, none diagnosed as HCC. **Figure 1** shows a typical run of AFPmRNA.

5. Discussion

Patients with liver cirrhosis were studied and followed up in this research, together with patients with HCC and healthy controls. The mean age of patients with LC was significantly different from patients with HCC, but not significantly different from healthy controls. This could be explained by the fact that HCC develops on top of LC after about 20-30 years of initial infection with HBV or HCV (16).

Hemoglobin levels were significantly lower in the HCC group compared to patients with LC. This could be due to the late presentation of patients with this cancer, which was certainly accompanied by loss of appetite and anemia due to chronic disease(17).

ALP was not significantly different between LC and HCC; however it is the only liver enzyme that carries a significant risk when it is above certain cut-off level, which is in agreement with Hann HW *et al*, who found that ALP was associated with increased risk for HCC in patients with LC (18).

According to the previous study GGT enzyme also had a significant relationship with increased HCC risk and there was also a significant association between GGT and overall cancer incidence (19). This contrasts with our study which showed no significant relationship and this goes with other study which showed no association with cancer mortality (20).It is important to notice that GGT levels could be affected by factors like diet, environmental pollutants and xenobiotic (19).

The main cause of Liver cirrhosis in this study was HBV, in agreement with another study which indicated that HBV is of high seroprevalence in Sudan (21). HCV infection was the third cause of LC in our patients, which is similar to results of a study carried out at the National Center for Gastrointestinal and Liver Disease in Khartoum, which concluded that HCV was of low seroprevalence (2.3%)(22).

Alcohol consumption was found to be the second cause of liver cirrhosis among the group, with 28% of patients acknowledging intake of alcohol, which goes with other studies in countries like USA where alcohol consumption is very high and more common than hepatitis C (23).

AFPmRNA was not detected in any of patients with liver cirrhosis without HCC, which is similar to previous study conducted by Liu Y *et al*(14). However, it was detected only in six patients of hepatocellular carcinoma. This is comparable to other studies where AFPmRNA was detected in 13.3%, 13.6%, 43.8% patients with liver cirrhosis (11, 24, 25) respectively. AFPmRNA was markedly expressed in HCC patients compared to patients without HCC (11).

Detection rate of HCC by AFP in this study was 64% which is in concordance with other studies where AFP detection rates range from 41 to 69% (8, 26, 27).

A close relationship exists between the expression of AFPmRNA and HCC development, and metastasis [30]. Using nested RT-PCR, AFPmRNA was detected in 24% of our patients with HCC. These results are similar to previous studies where AFPmRNA was detected in 25% of HCC cases (24), but is low compared to other results where the detection of AFPmRNA was ranged between 30% and 73.3% (11, 28-32). The detection rate of AFPmRNA increased to 66.7% where AFP level were higher than 200ng/dl and this represents the only significant relationship between the two biomarkers, which is in line with another study(28).

Most cases of HCC in whom AFPmRNA was detected were HBV seropositive (66.7%) compared to a previous study which indicated no association between AFPmRNA and hepatitis B infection (14). This is probably due to the high seroprevalence of HBV in Sudan.

In **conclusion**, this study has shown that combination of AFP, AFPmRNA and U/S increases sensitivity for the detection of early HCC nodules rendering them the method of choice for diagnosis of HCC on top of cirrhosis. AFPmRNA alone carries a poor detection rate for the diagnosis of HCC.

Table 1: Demographic information of enrolled patients

Group	Sex M (%)/ F (%)	Age (range)	HBV^{+ve} (%)	HCV^{+ve} (%)	Alcoholic (%)	No
HC	34 (68) /16 (32)	(37 - 58)	0	0	0	50
HCC	17 (68) /8 (32)	(47 - 70)	11 (44)	4 (16)	5 (20)	25
LC	78 (78)/22 (22)	(38 - 60)	42 (42)	6 (6)	28 (28)	100

Key: M= Male; **F**= Female; **HC**= Healthy Control;**LC**= Liver Cirrhosis; **HCC** = Hepatocellular carcinoma; **HBV^{+ve}** = Positive HBV; **HCV^{+ve}** =Positive HCV
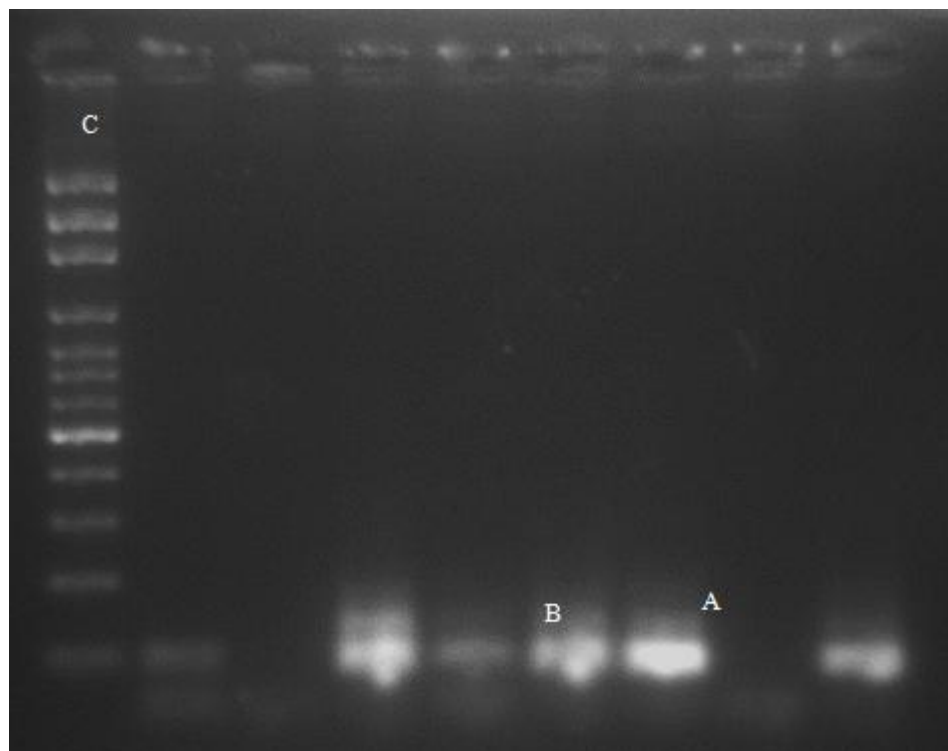

Figure 1: AFPmRNA detected in blood of patients with HCC. (PCR product (101 bp) of AFPcDNA using nested RT-PCR).
Key: A=Control Sample; B= AFPcDNA (101); C= DNA stepladder

Table 2: Results of liver function tests, AFP and haemoglobin (Can you use (SD) for AFP, please?)

Groups	Alb (±SD)	ALT (±SD)	AST (±SD)	GGT (±SD)	ALP(±SD)	AFP(±SD)	Hb(±SD)
H C	4.37(0.6)	8.9(5.4)	10.0(5.7)	7.65 (4.2)	7.5(4.6)	2.3(1.3-4.8)	14.6(1.5)
HCC+ve	3.0(0.4)	36.1(2.6)	68.5(13.1)	36.1(1.5)	38.0(4.5)	1000.0(648.3-1052)**	9.7(2.2)
HCC--ve	3.3(0.5)	93.6(10.1)	103.1(8.2)	66.1(5.1)	46.0(3.6)	36.2(4.0-122.8)	10.7(1.4)
LC	3.2 (0.8)	40.6 (38.7)	57.2 (5.4)	37.4(2.9)	31.5 (3.8)	5.3(2.7-14.4)	12.2 (2.1)

Key: **HC**= Healthy Control;**LC**= Liver Cirrhosis; **HCC** = Hepatocellular carcinoma; **Alb**= Albumin; **ALT** = Alanine minotransferase; **AST**= Aspartate aminotransferase; **GGT** = Gamma-glutamyl transferase; **ALP** =Alkaline phosphatase; **AFP** = alpha fetoprotein; **Hb**= Haemoglobin,

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