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Indicators of Hepatocellular Carcinoma in Sudanese Patients with Liver Cirrhosis

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DEDICATION

To my family
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ABBREVIATIONS:

HCC: Hepatocellular Carcinoma
LC: Liver Cirrhosis
AFP: Alpha Fetoprotein
AFPmRNA: Alpha Fetoprotein messenger RNA
ELISA: Enzyme Linked Immunosorbent Assay
HBV: Hepatitis B Virus
HCV: Hepatitis C Virus
Cum Hazard: Cumulative Hazard
HR: Hazard Ratio
CI: Confidence Interval
OR: Odd Ratio
RR: Relative Risk
TMB: Tetramethylbenzidine
ABSTRACT

Introduction:

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 Alpha-fetoprotein (AFP), ultrasound (U/S), alpha-fetoprotein messenger RNA (AFP mRNA) in addition to other indicators as early predictors for hepatocellular carcinoma (HCC) in Sudanese patients with liver cirrhosis (LC).

Material and 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 liver cirrhotic patients in addition to abdominal U/S for 3 years. Serum AFP was determined using ELISA in patients and controls. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used for detection of AFPmRNA in patients and controls.

Results:

During follow up hepatic nodules were identified in three patients (3%). AFP level and Alkaline phosphatase (ALP) enzyme were found to carry a high risk association with HCC in patients with LC (P<0.001, P=0.039) respectively. Significant differences were found between levels of liver enzymes and AFP in patients with HCC compared to patients with LC.
A significant difference was found in the level of (ALP) between healthy controls and LC (P<0.01). Significant differences were also found between patients and controls for the other enzymes. AFP levels kept increasing during follow up of patients with LC, but the increase was not significant (P=0.632). Regarding liver enzymes there was a significant decrease in AST level in patients with LC during follow up (P<0.001), and ALT levels (P=0.004). The same significant decrease was also found for GGT in the same group (P=0.002). However, ALP enzyme level did not change significantly with time (P=0.210). AFP sensitivity was higher at cut-off point (20ng/dl) which was 64% and specificity was 98%, the sensitivity of ultrasound alone was 76% but it increased to 84% when it was accompanied with AFP. AFP mRNA has a very low sensitivity (24%) but with high specificity.

**Conclusions:**

The study showed that the combination of U/S and AFP would be the most effective tool for HCC screening in Sudan in addition to ALP enzyme. However, AFP mRNA appears to have the lowest sensitivity and so it is not recommended for screening for HCC.
المقدمة:
تليف الكبد هو حالة منتشرة في السودان والعامل المسبب الرئيسي لسرطان الكبد.

الأهداف:
هذه الدراسة تقدم فعالية الفيتوبروتين (AFP)، الموجات الصوتية (ultrasound)، الموظفات النووي الريبي (AFPmRNA) بالإضافة إلى مؤشرات أخرى كمتيتات تطور سرطان الكبد في المرضى السودانيين الذين لديهم تليف في الكبد.

المؤاد والاساليب:
منة من المرضى السودانيين الذين لديهم تليف في الكبد. 25 مريض لديهم سرطان كبد و 50 من الأصحاء تم تضمينهم في الدراسة. مصل ألفا فيتو بروتين (AFP)، ANRmPFA و انزيمات الكبد تم قياسها سنويا في المرضى تليف الكبد بالإضافة إلى الموجات فوق الصوتية لمدة ثلاث سنوات. تم الكشف عن ه AFPmRNA مصل تم قياسه باستخدام ELISA في المرضى والضوابط. باستخدام PCR-RT في المرضى والضوابط.

النتائج:
خلال المتابعة تم تحديد العقيدات الكبد في ثلاثة مرضى (3%). مستوي ALP و إنزيم PFA تناسب مع زيادة خطر تطور سرطان الكبد في المرضى المصابين بتليف الكبد على النتائج P=0.039. (P<0.01) تم العثور على اختلافات كبيرة بين مستويات إنزيمات الكبد الفيتوبروتين في مرضى سرطان الكبد مقارنة مع تليف الكبد. تم العثور على اختلاف كبير في مستوي إنزيم ALP بين الأصحاء و مرضى تليف الكبد (P<0.01). ، تم العثور على فروقات ذات دالة إحصائية بين المرضى والكحترول لإنزيمات أخرى. ألفا فيتوبروتين استمر في الزيادة أثناء متابعة مرضى تليف الكبد ولكن كانت الزيادة ليست كبيرة (P=0.632).

ومعما يتعلق بإنزيمات الكبد كان هناك انخفاض ملحوظ في متوسط مستوي إنزيم AST خلال متابعة أقل (P=0.004) أيضا بالنسبة لGGT مرسي تليف الكبد، (P<0.001) كذلك في مستوى ALT لفط النمو مع الوقت لفط النمو مع الوقت (P=0.002) بينما لم يتغير مستوى إنزيم ALP بشكل ملحوظ مع الوقت. كانت نسبة مصابة بالمرضى كانت 20ng/dl وهي 64% والخصوصية كانت Afp حساسية Afp كانت أعلى عند نقطة قطع AFP و率为 0.210 (P=0.002).
(98%) حساسية الموجات الصوتية بمفردها كانت (76%) لكن ازدادت إلى (84%) عندما ترافقت مع AFP. كما ان (24%) لديه حساسية منخفضة (AFPmRNA) لكن لديه خصوصية عالية.

الاستنتاج:

أظهرت الدراسة أن الجمع بين الموجات فوق الصوتية و AFP سيكون الأختبار الأكثر فعالية لفحص سرطان الكبد. في السودان بالإضافة إلى إنزيم ALP. بينما لديه حساسية منخفضة لفحص mRNA AFP. وبالتالي ليس من المستحسن استخدامه لفحص مرضى سرطان الكبد.
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Chapter One
Introduction and Literature Review

1.1 Epidemiology of Hepatocellular Carcinoma:

Hepatocellular carcinoma (HCC) occurs mostly on top of liver cirrhosis (LC) and chronic liver inflammatory conditions. The geographic distribution of this cancer depends on the distribution of its predisposing factors like hepatotropic viruses (which cause 57-78% of cirrhosis and HCC cases). Hepatitis B virus (HBV) is the main cause of LC and HCC in Africa with approximately 18% of the global burden of HBV infection. HCC and LC accounting for 2% of the continent's annual deaths. Aflatoxin B1 consumption is added to HBV infection as the main predisposing factors in Sub-Saharan and West Africa, in addition to high alcohol consumption. HBV is also responsible for the endemicity of the cancer in China. Southeast Asia and other developing countries (more than 80% of HCC cases), although in countries like Taiwan this cancer is declining due to expanded vaccination programs.

Hepatitis C virus (HCV) infection is the main predisposing factor in Western Europe and North America in addition to excess alcohol consumption. This is also applied to Japan and most developed countries. In Japan, HCC occurs in 81% of HCV and 14% of HBV carriers, but seldom in alcohol-related liver cirrhosis, whereas in the United States HCC occurs in 30% of alcohol-related Liver cirrhosis.

Hemochromatosis is distributed according to ethnical background and is a predisposing factor for HCC. Other factors like diabetes mellitus type 2...
and obesity are taking more roles in the modern life as risk factors for non-alcoholic fatty liver diseases which predispose to liver cirrhosis and cancer. Sex is another important factor as males are more vulnerable to develop this cancer than females.

1.2 HCC in Sudan

HBV and peanut butter consumption are considered major risk factors for development of HCC in Sudan and 80% of HCC cases could be attributed to these two factors, if each factor is taken individually 27-60% of all cases are due to aflatoxin exposure, 49-52% to HBV infection and 7-34% is due to both factors.

Regarding contamination of peanut products with aflatoxin B1 in different regions of Sudan, a high contamination rate was found in western Sudan making it a high risk area for HCC. This is due to humid storage conditions compared to low contamination in central Sudan; a low risk area for HCC. Genetically polymorphic enzymes greatly influence the metabolism of this toxin and consequently the development of HCC. Strong relation was found between glutathione S transferase mu 1 gene (GSTM1) null genotype and the development of HCC, while glutathione-S-transferase theta 1 gene (GSTT1) polymorphism was found to have no relation with the development of this cancer.

Regarding viral infections HBV is considered to be of high seroprevalence in Sudan, with exposure to the virus ranging between 47-78%. But we have to notice that most studies were done before the referendum and separation of Southern Sudan. Some studies indicated that there was high seroprevalence in Southern Sudan 26% and low seroprevalence in central Sudan 6.8%, compared to other results that
suggested a higher HBsAg seroprevalence among asymptomatic blood donors of up to 17.5% in central Sudan\textsuperscript{20}.

Also, a high seroprevalence of HBsAg was observed among liver cirrhotic patients (31-61\%)\textsuperscript{21,22} with carrier rates between 43-60\% in patients with HCC\textsuperscript{22,23}. In patients with end stage renal disease the seroprevalence was 7.6\%\textsuperscript{24} but the new trend to vaccinate patients planned for hemodialysis if they are negative for HBV antibodies will decrease the infection rate\textsuperscript{16}. A more recent study confirmed the low seroprevalence of HBsAg among hemodialysis patients in Ahmed Gasim hospital, Khartoum North was (4.5\%) which could be attributed to vaccination programs in Khartoum state\textsuperscript{25}.

Other more vulnerable groups to this viral infection comprise doctors, 97.2\% nurses, 98.6\% lab technicians 94.8\%, and other health workers 95.7\%, who have a good knowledge about the methods of transmission of this virus. Paramedical know that HBV was transmitted via blood, but actually they don’t apply this knowledge to the practical ground as only 33\% of doctors were always wearing gloves and more than 50\% of health workers are not vaccinated and had poor knowledge about Universal Standard Precautions Guidelines\textsuperscript{26}.

HBV is considered to be one of the commonest predisposing factors for HCC in Sudan but this is not the fact regarding HCV which was found to be of low seroprevalence in Sudan compared to Egypt which records a high infection rate of HCV that approaches 20\% of villagers above 20 years\textsuperscript{27}. This is attributed to mass treatment with parenteral antischistosomal therapy,\textsuperscript{28} In Sudan, a low seroprevalence of 2.2\% was demonstrated in Gezira area which was endemic region for schistosomiasis\textsuperscript{29}. Genotype 4 was the commonest isolated genotype,\textsuperscript{30} but no association was found between HCV infection
and schistosomiasis or with parenteral antischistosomal therapy. Among asymptomatic blood donors, the prevalence of HCV was found to be 4.4% as compared to the high infection rates among patients with end stage renal disease on hemodialysis 23.7%. More high seroprevalence of 34% was recorded in older study conducted on 1994. Longer dialysis duration, previous surgery, age of more than 30 and multiple dialysis center, were all associated risk factors.

About 57% of all HCC cases in Sudan could be attributed to HBV and HCV infection.

1.3 Molecular basis of hepatocarcinogenesis

HCC often develops on top of chronic liver inflammatory condition or liver cirrhosis. During preneoplastic phase the continuous regeneration and degeneration of cells leads to dysplastic hepatocytes foci which are finally transformed to cancer cells due to genetic and chromosomal aberration enhanced by growth factors, inhibitors and triggers. HCC is characterized by an obvious multistage process of tumor progression from a regenerative nodule to adenomatous hyperplasia, then to atypical adenomatous hyperplasia, early HCC (defined as in situ or micro invasive cancer), and advanced HCC.

The mean size of most dysplastic nodules or adenomatous hyperplasia is 7-8 mm, rarely larger than 20 mm in diameter.

Hepatocyte microsatellite instability occurs in chronic hepatitis, cirrhosis and HCC. Examination of multiple loci detects allelic deletions in 30%-50% of chronic hepatitis or cirrhosis cases, in 70%-80% of dysplastic nodules and in almost all HCC cases. It is suggested that although many cells have early genomic aberrations in chronic liver disorders, they do not evolve necessarily into the malignant phenotype.
Some studies found that Apoptosis resistance and uncontrolled proliferation of hepatocytes are associated with increased expression of c-Jun, a redox-sensitive transcription factor. C-Jun affects apoptosis by preventing cell death, enhances hepatocyte cells proliferation and stimulates expression of AFP; a biomarker for development of HCC in patients with liver cirrhosis. Also tumor suppressor genes mutations like P53 gene and Rb gene are associated with increased risk of hepatocarcinogenesis. It is also found that some HBV and HCV genotypes are more involved in hepatocarcinogenesis process than others. For example, the HCV subtype 1b (HCV/1b) is associated with more severe clinical outcome and poor antiviral response. Moreover, patients infected with HBV genotype C (HBV/C) present a higher frequency of HCC than HBV/B-infected patients. In the same way, a double mutation (A1762T/G1764A) in HBV basal core promoter (BCP) region has been implicated with severe clinical outcome and poor response to nucleosides analogues.

1.4 Risk factors for HCC

1.4.1 Liver Cirrhosis

C is a precancerous condition and is defined as the histological development of regenerative nodules surrounded by fibrous bands in response to chronic liver injury that leads to portal hypertension and end stage liver disease. The prevalence of LC worldwide is not well known because compensated cirrhosis often goes undetected for prolonged time but it is estimated that 1% of population have LC. The pathological process is complicated and results from imbalance between synthesis and degradation of extracellular matrix leading to replacement of normal parenchymal cells with connective tissues, and the main predisposing factors are HBV, HCV, alcohol
consumption and autoimmune diseases\textsuperscript{38}.

1.4.2 Alcohol consumption

Alcohol consumption increases the risk of developing HCC\textsuperscript{55}. It is known that ethanol is metabolized by alcohol dehydrogenase and aldehyde dehydrogenase, both enzymes have polymorphic forms that influence the conversion of ethanol to acetaldehyde and some of these polymorphic forms were shown to increase alcoholic liver disease\textsuperscript{56}.

Cytochrome P450 (CYP 2E1) is mainly involved in ethanol metabolism and inhibition or induction of the activity of this microsomal oxidizing system was found to be correlated with alcohol induced liver disease\textsuperscript{57}. It is assumed that alcohol metabolism causes oxidative stress and formation of reactive oxygen species which eventually leads to DNA damage. Also alcohol increases the absorption of iron from the gut which accumulates in the liver and enhances lipid peroxidation\textsuperscript{58}. Other effect of alcohol consumption is related to vitamin A which has important role regarding proliferation and growth of cells. Alcohol leads to a decreased level of this vitamin in liver\textsuperscript{59}.

A close relationship was found between alcohol consumption and co-infection with hepatotropic viruses and HCC. Regarding HBV infection many studies have found high hepatitis B markers in patients with alcohol related HCC\textsuperscript{60}. Also HCV was found to aggravate the histological damage of hepatic cells leading to fast progression to HCC in alcoholic patients\textsuperscript{61}.

1.4.3 Hepatitis B Virus (HBV)

HBV is a circular DNA virus that can randomly integrate into human genome inducing chromosomal instability resulting in rearrangement or deletion such as insertional mutations at specific sites\textsuperscript{62}. Up to 5\% of adults
and 80% of infants infected with HBV become chronic carriers, and approximately 10% of these develop chronic hepatic inflammation, hepatocellular degeneration and necrosis, finally resulting in liver cirrhosis. HCC develops in about 5% of chronic carriers with or without cirrhosis 20–30 years after initial infection $^{63,64}$.

Molecular mechanisms leading to HCC in chronic HBV infection has been extensively studied. One of the important viral proteins implicated is hepatitis B x protein (HBx) which is a multifunctional regulatory protein that communicates directly or indirectly with a variety of host targets, and mediates many opposing cellular functions, including cell cycle regulation, transcriptional regulation, signaling, encoding of the cytoskeleton, cell adhesion molecules, as well as oncogenes and tumor suppressor genes $^{65}$. Other factors included are HBV genotype C and the basic core promoter (BCP) mutations which reported to be associated with the development of HCC $^{66}$.

The role of cytokines in liver fibrosis and cell proliferation is added to the several HBV genes that have been found in infected tissues, including truncated pre-S2/S, hepatitis B X gene, and hepatitis B spliced protein. The proteins expressed from these genes have been shown to affect cellular growth and apoptosis $^{67}$. Also some patients with HCC have no detectable hepatitis B surface antigen in their serum but have low levels serum HBV DNA and integrated molecules of HBV DNA in their tissues. Occult HBV infection may account for a proportion of cases of HCC that occur in patients without serologic markers for hepatitis B and C and may be a cofactor in HCC in patients with chronic hepatitis C who have coexistent occult HBV infection $^{67}$. 
1.4.4 Hepatitis C virus (HCV)

Over 200 million people are estimated to be infected with HCV worldwide. Within the United States, HCV infection is the leading cause of chronic hepatitis and cirrhosis, and is an increasingly important factor in the etiology of HCC.\textsuperscript{68} The increase in incidence of HCC worldwide in the past several decades was attributed to the increase in the number of patients chronically infected with this virus\textsuperscript{69, 70}. Cirrhotic patients infected with HCV develop hepatic decompensation at a rate of 30\% over 10 years and hepatocellular carcinoma at annual rates ranging from 3\% to 8\%\textsuperscript{134}.

HCV infection results in chronic hepatic inflammation, degeneration and necrosis, followed by cirrhosis, but with much higher frequency than in the case of HBV infection: 60–80\% of patients with acute hepatitis due to HCV infection develop chronic hepatitis, and HCC occurs in approximately 20\% of patients with chronic HCV infection 20–30 years after the initial infection\textsuperscript{71, 72}. Although cirrhosis and hepatocellular carcinoma can arise in persons with chronic hepatitis C, these outcomes do not always occur. The cirrhosis risk is greater in those who are infected at older ages, those who drink >50 g of alcohol per day, and persons coinfected with HIV\textsuperscript{73}.

Oncogenic potential of the virus could be explained by interacting with many host-cell factors and affecting a wide range of activities including cell cycle regulation, transcriptional regulation, cell proliferation, apoptosis, lipid metabolism, and cell growth promotion. It is also suggested that HCV contributes to HCC by modulating the pathways that may promote malignant transformation of hepatocytes. At least four of the 10 HCV gene products;
namely core, NS3, NS5A and NS5B play roles in several potentially oncogenic pathways. Induction of Endoplasmic reticulum (ER) stress and oxidative stress by HCV proteins may also contribute to hepatocyte growth promotion\textsuperscript{74}.

1.4.5 Aflatoxin B1

Aflatoxin (AFB), a mycotoxin from Aspergillus flavus; can damage hepatocyte DNA after its conversion into exo-8, 9-epoxide by cytochrome p450. After this conversion, AFB-8, 9-epoxide reacts with guanine nucleotides to form a connection which induces a genetic mutation. Moreover; aflatoxin can alter p53 control of cell proliferation\textsuperscript{75}. It is well established that HCCs developing in patients highly exposed to aflatoxin B1 frequently contain a specific G to T transversion in codon 249 of the p53 gene, whereas HCC from areas without significant exposure to this carcinogen do not contain specific p53 mutations\textsuperscript{76,77}.

1.4.6 Other factors

Other factors that contribute to liver cirrhosis and HCC is hereditary hemochromatosis in which hepatic iron overload often results in fibrosis and cirrhosis and may be complicated by the development of HCC. The mechanism by which iron induces malignant transformation is by the generation of oxidative stress. Oxidative stress leads to lipid peroxidation of unsaturated fatty acids in membranes of cells and organelles. Free iron also induces immunologic abnormalities that may decrease immune surveillance for malignant transformation\textsuperscript{78}.

HCC has also recently been linked to metabolic conditions like non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of obesity and related metabolic disorders such as diabetes. There is also evidence that
NAFLD acts synergistically with other risk factors of HCC such as chronic hepatitis C and alcoholic liver injury. Major systemic and liver-specific molecular mechanisms involved include insulin resistance and hyperinsulinemia, increased TNF signaling pathways, and alterations in cellular lipid metabolism\textsuperscript{79}.

1.5 Tumor markers for HCC

Early detection of HCC is important as early treatment by surgical resection, liver transplantation and percutaneous ablation could be lifesaving,\textsuperscript{82} many tumor markers have been suggested for this purpose including fucosylated fraction of AFP (AFP-L3) fraction\textsuperscript{80, 81} and prothrombin induced by vitamin K absence-II (PIVKA-II) or Des-\textgammacarboxyprothrombin\textsuperscript{83}. However, diagnostic values of these markers are controversial and different cut-off values have been suggested depending on the study design\textsuperscript{82}.

1.5.1 AFP and ultrasound for surveillance of HCC:

Currently, the recommended screening strategy includes measurement of serum AFP levels and an abdominal ultrasound every 3-6 months for the detection of HCC at an earlier stage\textsuperscript{84 – 87}. Usually, a serum AFP level of 20 ng/dL is considered as a cut-off value to differentiate HCC from non-HCC\textsuperscript{88}. Compared with no surveillance, this strategy is estimated to be more than triple the number of patients with operable HCC tumors at time of diagnosis, and almost half the number of deaths from HCC\textsuperscript{89}. Many studies indicated that sensitivity of combined AFP and ultrasound increase with small drop in specificity and so it is the best strategy to detect HCC at an early stage\textsuperscript{90}.

The benefit of HCC surveillance has been demonstrated by a randomized controlled trial performed in chronic HBsAg carriers, and confirmed by several cohort studies carried out in cirrhotic patients\textsuperscript{91}.
Semiannual surveillance is superior to annual surveillance in terms of cancer stage, amenability to curative/effective treatment and patient’s survival. Moreover, a semiannual U/S based surveillance is cost effective. Nonetheless in the USA, surveillance for HCC is carried out in less than 20% of patients at risk, and in Italy only a half of HCCs are diagnosed during surveillance. The study by Singal et al. demonstrates that poor patient’s compliance seems to be one of the main reasons for the low yield of surveillance in clinical practice, as almost 40% of individuals inconsistently followed, or did not follow the recommended surveillance schedule. Recently, a meta-analysis has shown that the sensitivity of U/S alone for the identification of early stage HCCs is 63%, and this figure is increased by 6% adding AFP measurement.

In Sudan the recommended surveillance program by combined AFP and U/S is followed up in Ibn Sina hospital but no available data to evaluate the effectiveness in Sudanese patients with liver cirrhosis, which the main objective of this study.

1.5.2 Molecular Markers for HCC

A large number of molecular factors have been shown to associate with the invasiveness of HCC, and have potential prognostic significance. One important aspect is the analysis of molecular markers for the cellular malignancy phenotype. These include alterations in DNA ploidy, cellular proliferation markers, nuclear morphology, the p53 gene and its related molecule MD M2, other cell cycle regulators (cyclin A, cyclin D, cyclin E, cdc2, p27, p73), oncogenes and their receptors, apoptosis related factors (Fas and FasL), as well as telomerase activity. Another important aspect is the analysis of molecular markers involved in the process of cancer invasion and
metastasis. Adhesion molecules (E-cadherin, catenins, serum intercellular adhesion molecule-1, CD44 variants), proteinases involved in the degradation of extracellular matrix. Tumor angiogenesis is critical to the growth and metastasis of cancers and many angiogenesis-related markers, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived endothelial cell growth factor (PD-ECGF), thrombospondin (TSP), angiogenin, pleiotrophin, and endostatin (ES) levels, as well as intratumor microvessel density (MVD) have been evaluated and found to be of prognostic significance.  

1.5.3 AFP-mRNA as molecular marker for HCC

AFP, a 70 kD glycoprotein synthesized from fetal yolk sac, liver and intestines. It has a half-life of 5-7 days. Serum AFP is a prognostic indicator of the response and survival of germ cell tumors. However, when an AFP level is slightly elevated it may be falsely elevated due to non-neoplastic liver disease. Recently, the separation of AFP-mRNA from peripheral blood mononuclear cells by RT-PCR has been extensively studied which may improve the effectiveness in screening of HCC patients. It is suggested that there is a very close relationship between the expression of AFP-mRNA and HCC occurrence, development, metastasis and prognosis. Circulating AFP-mRNA has been proposed as a marker of HCC cells disseminated into the circulation. The specificity of this molecular marker and its correlation with the main HCC clinico-pathological parameters remains controversial. However, the detection of HCC cells in the peripheral blood is not only important for the diagnosis of HCC, but is also of critical importance for early detection and treatment of HCC.
AFP-mRNA was found to improve sensitivity and specificity of AFP and is a useful marker to diagnose HCC or monitor metastasis and relapse. This is added to the finding that the human AFP gene is known to be transcribed prominently in HCC, but weakly in normal adult hepatocytes. Using nested RT-PCR assay, circulating AFP-mRNA could be detected in HCC patients without clinical evidence of extra-hepatic metastasis as well as in patients with liver cirrhosis.

1.6.1 Rationale
High incidence of HCC in Sudan that carries a dismal prognosis and low survival rates that needs more effort to explore the predisposing risk factors and to evaluate more predictive biomarkers that can help to improve the bleak outlook for this killing malignancy.

1.6.2 General objectives
To evaluate the use of ultrasound (U/S), AFP and AFP-mRNA as early predictors of HCC in Sudanese patients with liver cirrhosis.

1.6.3 Specific objectives
1- To measure serum AFP in Sudanese patients with liver cirrhosis as predictive biomarker for hepatocellular carcinoma.
2- To detect early nodules for hepatocellular carcinoma in patients with liver cirrhosis by ultrasound.
3- To detect AFP mRNA in Sudanese patients with liver cirrhosis as predictor for HCC.
4- To find associations between factors like age, sex, HBV, HCV and alcohol consumption and the development of HCC in the same group.
CHAPTER TWO
MATERIALS AND METHODS

2.1.1 Study area

This study was carried out at Ibn Sina hospital (National Center for Gastrointestinal and Liver Diseases), Khartoum, Sudan; which acts as a reference center serving areas all over the country especially the capital city hospitals from which all patients are referred.

2.1.2 Study design:

The study is a prospective case control study in which 100 patients (males and females) with liver cirrhosis were followed up for 36 months, in addition to 25 patients with HCC and 50 healthy controls, age matched and sex matched.

2.1.3 Study population

All adult patients with liver cirrhosis attending the clinic at Ibn Sina hospital were enrolled in the study if they fulfill the following criteria: (1) they had a confirmed diagnosis of liver cirrhosis by physical examination, laboratory measurements and ultrasonography; (2) they were in good clinical condition (Child's grade A or B\textsuperscript{103}); (3) they were at least 20 years old (as recommended by American association for the study of liver disease (AASLD\textsuperscript{102}); (4) they have no known or suspected hepatic neoplasm; and (5) they were willing to cooperate by visiting the clinic at scheduled intervals. Patients with Child's grade C disease were excluded on the assumption that these patients were unlikely to survive long enough to allow meaningful evaluation, and their surveillance will be pointless\textsuperscript{103}. 

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Patients with liver cirrhosis were classified to the following categories due to the cause of cirrhosis:

(1) Hepatitis B virus — this category included patients with a positive serum test for hepatitis B surface antigen
(2) Hepatitis C virus — patients seropositive for antibody for hepatitis C virus
(3) Alcoholism — a daily intake of traditional wine for more than 10 years
(4) Multiple causes — this category included patients with a combination of two or more of the above causative factors
(5) Other causes — this category included patients with none of the above causative factors.

The patients were followed up by AFP, U/S scanning which were repeated at yearly intervals by an experienced sonographer at Ibn Sina hospital in all cirrhotic patients in addition to AFP-mRNA.

The diagnosis of HCC in patients with liver cirrhosis depended on AASLD recommendations (2005) to the diagnosis, staging and treatment of patients diagnosed with HCC, where detection of a hepatic mass within a cirrhotic liver is highly suspicious of HCC, if AFP is greater than 200 ng/dL and the radiological appearance of the mass is suggestive of HCC (large and/or multifocal disease with arterial hyper vascularity), the likelihood that the lesion is HCC is high and biopsy is not essential.

The routine evaluation of the patients included taking a medical history, performing a physical examination and a complete blood count, and recording liver biochemical tests (like: Serum albumin, liver enzymes, bilirubin and INR). Abnormal baseline enzyme levels were defined as the following: for alanine aminotransferase (ALT), the upper limit of normal (ULN) range is ≤40
U/L for males and ≤31 U/L for females; for aspartate aminotransferase (AST), the ULN range is ≤37 U/L for males and ≤31 for females; for alkaline phosphatase (ALP), the ULN range is ≤117 U/L for males and females. For gamma gultamyl transferase (GGT), the ULN range is ≤51 U/L for males and ≤33 U/L for females.

**HCC group:** Included 25 patients who were diagnosed with HCC on top of liver cirrhosis male and female sex from the same age group.

**Control group:** Consisted of 50 healthy subjects sex and age matched without liver disease from the same age group and community of the patients who have been randomly chosen.

**Data collection:** This was obtained by using a questionnaire, clinical examination and laboratory investigations. Questionnaire included information about the patient sex, age, occupation, habits, alcohol consumption and past medical history.

The following formula was used for calculation of the sample size:

\[ n = \frac{t^2 \times p(1-p)}{m^2} \]

(n= required sample size, \( t \) = confidence level at 95% (standard value of 1.96), estimated prevalence of liver cirrhosis in Sudan, \( m \) = margin of error at 5% (standard value of 0.05))

**2.1.4 Ethical consideration**

This study was approved by the Ethical Committee, Faculty of Medicine, University of Khartoum. Verbal consent has been taken from all patients and controls and they were informed about all their rights without affecting their right in medical care. They were informed that they were free to withdraw at any time.

**2.2.1 Methods**
2.2.1 Serum alpha fetoprotein

Five ml of venous blood sample were obtained by venipuncture from each patient using disposable syringe. The blood was drained in plain and heparinized tubes and the serum or plasma was separated by centrifugation. The specimens were aliquoted and deep frozen at -40c until analysis time. The serum was used for estimation of AFP using enzyme immunoassay (ELISA). The method is based on one step sandwich ELISA\textsuperscript{104}.

2.2.2 A Principle of the test

Standard sandwich ELISA procedure was followed, AFP antibody coated beads and the enzyme labeled antibody were incubated with the serum. Then AFP in the serum was sandwiched between the other two constituents. The measurement of the enzyme activity provides a quantitation of AFP in the specimen so the amount of AFP bound to the solid phase is eventually reflected by the amount of the enzyme.

20µl of standard, specimens and controls were dispensed into appropriate wells. 100 µl of zero buffer according to the kits manual (Immunospec Corporation. Cat no.E29-205) was added to each well and thoroughly mixed. Samples were incubated at room temperature for 30 min, and then 150µl of enzyme conjugate reagent was dispensed into each well. After incubation the microtitre wells were rinsed with washing buffer and another 100µl of TMB (Tetramethylbenzidine) substrate was added and mixture was incubated for 20 mins. The reaction was stopped by adding 100µl of (2N HCL) solution and the blue color changed to yellow. The optical density was read at 450nm with a microtitre reader (Biotek instrument.USA.SN 214375). The unknown sample concentration was read from the standard curve (Figure 3.1).

2.2.3 Detection of AFP-mRNA
Heparinized whole blood was centrifuged and plasma fraction was removed. The cellular fraction was enriched for mononuclear cells or possible tumor cells according to the method described by Komeda\textsuperscript{105}.

RNA was extracted by qiagen-RNeasy midi kit. Nested RT-PCR was conducted by adding master mix: Rnase-free water 11µl, qiagen one step RT-PCR buffer 5 µl, dNTPs mix (containing 10MmM of each dNTPs) 1µl, EX-sense 2µl, EX-antisense 2µl, qiagen one step RT-PCR enzyme mix 1µl, 22µl of the master mix were added to 3µl template RNA for each sample.

The reaction mixture was subjected to 35 cycles of amplification in a programmable thermal cycler, by using the following sequence: 94C° for 1.5 min, 57C° for 1.5 min and 72 C° for 2.5 min, plus a final extension step at 72C° for 10 min, a sample of 3µl of first amplification product was further amplified using an inner pair of primers (IN-sense and IN-antisense) (Promega Corporation. Cat no.G4521), to verify the amplified AFP DNA fragment. Samples were analyzed by 15% polyacrylamide gel electrophoresis and stained with ethidium bromide to identify the specific bands of 174 base pairs (first amplification product) and 101 base pairs (second amplification product). For samples with conflicted results nested PCR was repeated two or more times. The design of external and inner pairs of primers was as follows:

- EX-sense 5'-ACTGAATCCACAACACTGCATAG-3'
- EX-antisense 5'-TGCAGTCAATGCATCTTCACCA-3'
- IN-sense 5'-TGGAATAGCTTCCATATTGGATTC-3'
- IN-antisense 5'-AAGTGCGCTTTGAACAAACTGG-3'

PCR products of 174 and 101 base pairs were amplified from AFPcDNA by external and internal primer pairs, respectively.

The location of the primer pairs was as follows:
EX-sense in exon 1 (AFPmRNA nucleotides 90-112), EX-antisense in exon 2 (AFPmRNA nucleotides 263-241), IN-sense over exon 1 and exon 2 (AFPmRNA nucleotides 122-145), IN-antisense in exon 3 (AFPmRNA nucleotides 222-200)^106.

2.3 Statistical analysis

Statistical analysis was performed with SPSS software. The Data were tested statistically for normal distribution by (Kolmogorov-Smirnov & Shapiro-Wilk) tests and were expressed as mean ± standard deviation (SD). Differences between groups were assessed by Student's t-test or the Chi-square test. Abnormally distributed variables were compared by Mann-Whitney test. A P<0.05 was considered statistically significant. The association between liver enzymes, AFP and HCC risk were expressed by hazards ratio (HR) and 95% confidence interval (CI) that were estimated using the Cox proportional hazards regression model, Kaplan-Meier analysis was used to compare the cumulative risks of developing HCC in patients with different levels of serum liver enzymes and AFP, and log rank test was used to determine the statistical significance.

Sensitivity and specificity were also statically assessed, where:
Sensitivity = True positive / True positive + False negative X 100
Specificity= True negative / True negative + False positive X 100
Chapter Three

RESULTS

The general characteristics of the hundred patients with liver cirrhosis that were included in the study were shown in (Table 3.1) and a comparison between healthy controls, liver cirrhosis and HCC groups regarding different variables is shown in (Table 3.2).

Most of the patients with liver cirrhosis 78 (78%) were males (Figure 3.2), mainly in age group of (41-60) and representing (67%) of patients. The main cause of liver cirrhosis in this group was hepatitis B viral infection with 42 (42%) of patients followed by alcohol consumption 28(28%), then (other causes) which included: nine with autoimmune hepatitis, two with primary biliary cirrhosis (PBC), one with secondary biliary cirrhosis (SBC) and one with congenital liver cirrhosis. Hepatitis C viral infection represented 6 (6%) and multiple causes; which included two alcoholics patients with HBV, one alcoholic with diabetes, two diabetics infected with HBV, one co-infected with both HBV and HCV represented 6(6%) of all patients. The last group was liver cirrhosis of unknown cause (5%), (Figure 3.3).

There was a significant difference between the age of patients with HCC and those with liver cirrhosis (P<0.001). There was no significant difference between the ages of healthy controls and patients with LC (P>0.05). Hemoglobin (Hb) level was significantly different between the three groups (P<0.001). Albumin level was significantly different between healthy controls and LC patients (P< 0.001). However its level was not different between HCC and LC groups.
Total bilirubin was significantly different between healthy controls and LC (P< 0.001), but the difference was less significant between LC and HCC groups (P<0.05). Prothrombin time (INR) was also significantly different between the three groups (P<0.001).

During follow up three of the patients died, none of them was diagnosed with HCC before death. Four patients from southern origin were missed for follow up, other six patients were missed for follow up at one and two years, demographic and clinical characteristics of those patients were not different from the other patients included in the group.

Three patients developed HCC during follow up and their characteristics are shown in Table (3.4). The diagnosis depended on AASLD recommendations for diagnosis HCC on top of liver cirrhosis. Another alcoholic patient developed a solitary nodule >2CM, had a normal AFP level and no AFP-mRNA was detected but he didn’t give his consent for liver biopsy and so diagnosis was not confirmed.

Liver function tests were done to all patients with liver cirrhosis; AST was high in (58%) of patients, ALT (alanine transaminase) was high in (32%), GGT (gamma glutamyl transferase) was high in (26%), while ALP (alkaline phosphatase) was high in only (6%).

During follow up the level of liver enzymes decreased significantly, mean AST levels were as follows for the three years respectively (57.4 (±52.2), 42.12(±25.7), 35.3 (±20.5), P value< 0.001 , also a significant difference was found between ALT levels during follow up (40.7(±38.6), 32.7 (±23.15), 2012: 28.5(±16.3) .P value=0.004. the same was observed for GGT level (37.4(±21.9), 32.5(±15.7), 29.4(±12.3) P value=0.002. However no significant
difference was observed for ALP (31.5(±38.5), 28.7 (±28.8) , 26.2 (±21.9) , P value =0.210, (Table 3.5).

Regarding HCC risk there was no significant association between AST enzyme and increased HCC risk (P=0.741) neither a significant relation was found between ALT (P= 0.198) or GGT (P= 0.107) and increased risk for HCC, the only significant relation was found between ALP enzyme and increased HCC risk (P= 0.039), also no significant relation was found between any of the above liver enzymes and age of patients, neither a significant relation was found between liver enzymes and sex of patients, or between liver enzymes and AFP level or causes of liver cirrhosis, the only Significant relationship was found between AST and male sex (P-value=0.020), (Table 3.3).

There was no significant relation between age of patients and HCC risk (P=0.488) or gender and increased risk for HCC (P=0.355).

There was increase in level of AFP during follow up, but the change in level was not significant P value=0.632. There was strong relationship between AFP level and increased risk for HCC (P<0.001) (Figure 3.9). Also a significant relation was found between the level of AFP and the age of patients (P-value<0.001), but no significant relation was found between the sex and AFP level among liver cirrhotic group (P-value =0.355). Neither a relation was found between the cause of liver cirrhosis and the level of AFP (P-value=0.827), or between the age of the patients and the cause of liver cirrhosis (0.234), (Table 3.3).

Figure 3.9 shows a typical run of AFP-mRNA which was detected during follow-up in three patients who developed HCC. AFP-mRNA was not
detected in any of patients with liver cirrhosis without HCC even those who died or were missed during follow up.

**Hepatocellular carcinoma group**

Most of Patients with HCC on top of liver cirrhosis group were males (68%), (Figure 3.11), mainly in age group (>60yrs) 48% (Figure 3.12).

HCC was predisposed in most of patients in this group by HBV (44%) followed by alcohol consumption (20%), then HCV (16%), then (multiple) and (other) causes both of which represented (8%) of patients, and at last unknown cause which represented (4%) of patients (Figure 3.4).

Regarding liver enzymes, AST was elevated in 84% of patients, ALT was high in 56%, ALP in 36% and GGT in 52%.

No significant relation was found between any of the above liver enzymes and age of patients with HCC, neither a relation was found between liver enzymes and AFP level or cause of HCC , but strong relationship was found between elevated ALP level and female sex (P˂0.001), (Table 3.3).

No significant relation was found between level of AFP and the cause of HCC (P=0.577), but a significant relation was found between AFP level and age of patients (P-Value=0.035). Also no significant relation was found between AFP level and detection of AFPmRNA in blood of HCC group (P-Value=0.057), the only significant relation between the two variables was found at AFP level above 200ng/dl (P-Value<0.01), (Table 3.3).

AFP cut off level >20 ng/dl was chosen according to other studies, the sensitivity of AFP for detection of HCC tumor at this level is 64% and specificity is 98%. The sensitivity of ultrasound alone is 76% and specificity 100% but the sensitivity increase to 84% when both of AFP and ultrasonography were accompanied together, the sensitivity of AFPmRNA
alone is only 24% but the sensitivity increase to 66.67% in AFP level higher than 200ng/dl, 66.7% of patients with HCC in whom AFPmRNA was detected were HB +ve.
Table 3.1: Characteristics of patients with Liver cirrhosis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean (SD)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>47.4 (± 11.4)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td>78 (78%)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>22 (22%)</td>
</tr>
<tr>
<td>Causes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td></td>
<td>42(42%)</td>
</tr>
<tr>
<td>HCV</td>
<td></td>
<td>6(6%)</td>
</tr>
<tr>
<td>Alcoholic</td>
<td></td>
<td>28(28%)</td>
</tr>
<tr>
<td>Multiple</td>
<td></td>
<td>6(6%)</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>13(13%)</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>5(5%)</td>
</tr>
</tbody>
</table>
### Table 3.2: Comparison between different variables in healthy controls, patients with liver cirrhosis and HCC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (Mean ± SD)</th>
<th>LC group (Mean ± SD)</th>
<th>HCC group (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>49.86(8.6)</td>
<td>47.4(11.4)</td>
<td>58.4(10.9) ***</td>
</tr>
<tr>
<td>Hb</td>
<td>14.6(1.5)</td>
<td>12.209(2.1) ***</td>
<td>10.5(1.7) ***</td>
</tr>
<tr>
<td>Albumin</td>
<td>4.37(0.6)</td>
<td>3.218 (0.8) ***</td>
<td>3.452 (0.6)</td>
</tr>
<tr>
<td>AST</td>
<td>10.0(5.7)</td>
<td>57.2 (52.4) ***</td>
<td>94.8 (74.1) **</td>
</tr>
<tr>
<td>ALT</td>
<td>8.996(5.40)</td>
<td>40.6 (38.7) ***</td>
<td>79.9 (91.7) ***</td>
</tr>
<tr>
<td>ALP</td>
<td>7.52 (4.6)</td>
<td>31.5 (35.8) ***</td>
<td>44.1 (37.9)</td>
</tr>
<tr>
<td>GGT</td>
<td>7.65 (4.19)</td>
<td>37.4(21.9) ***</td>
<td>58.9 (45.9) ***</td>
</tr>
<tr>
<td>T.Bilirubin</td>
<td>0.656(0.299)</td>
<td>1.806(1.12) ***</td>
<td>2.33(0.986) *</td>
</tr>
<tr>
<td>INR</td>
<td>0.94(0.193)</td>
<td>1.379(0.459) ***</td>
<td>2.24(0.83) ***</td>
</tr>
</tbody>
</table>

Student's t test and Mann-Whitney Test were used to compare between different groups. Age (years), Albumin (g/dl), liver enzymes (IU/L), bilirubin (mg/dl). (*= (P<0.05), **= (P<0.01), ***= (P<0.001))
Table (3.3): correlations between different variables (P value)

<table>
<thead>
<tr>
<th></th>
<th>Sex (LC)</th>
<th>Age (LC)</th>
<th>Cause (LC)</th>
<th>Age (HCC)</th>
<th>Sex (HCC)</th>
<th>Cause (HCC)</th>
<th>AFP (HCC)</th>
<th>AFP (LC)</th>
<th>AFP (HCC) mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (LC)</td>
<td>-</td>
<td>-</td>
<td>P&lt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Age (LC)</td>
<td>-</td>
<td>-</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Cause (LC)</td>
<td>P&lt;0.001</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Age (HCC)</td>
<td>-</td>
<td>-</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&lt;0.001</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Sex (HCC)</td>
<td>-</td>
<td>-</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&lt;0.001</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Cause (HCC)</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFP (HCC)</td>
<td>P&gt;0.05</td>
<td>P&lt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFP (LC)</td>
<td>P&gt;0.05</td>
<td>P&lt;0.001</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFP (HCC) mRNA</td>
<td>P&lt;0.01</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>
Table (3.4): Age, sex and level of AFP among patients with liver cirrhosis who developed HCC during follow-up

<table>
<thead>
<tr>
<th>Cause</th>
<th>Age (yrs.)</th>
<th>Sex</th>
<th>AFP (ng/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-HBV</td>
<td>55</td>
<td>Male(1)</td>
<td>210.586</td>
</tr>
<tr>
<td>2-alcoholic</td>
<td>70</td>
<td>Male(1)</td>
<td>574.046</td>
</tr>
<tr>
<td>3-multiple (HBV+HCV)</td>
<td>53</td>
<td>Male(1)</td>
<td>398</td>
</tr>
</tbody>
</table>
Table 3.5: Changes in the levels of liver enzymes during follow-up

<table>
<thead>
<tr>
<th>Enzyme/time</th>
<th>AST*</th>
<th>ALT**</th>
<th>GGT***</th>
<th>ALP****</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st yr</td>
<td>57.4 (± 52.2)**</td>
<td>40.7(±38.6)</td>
<td>37.4(±21.9)</td>
<td>31.5(±38.5)</td>
</tr>
<tr>
<td>2nd yr</td>
<td>42.12(±25.7)*</td>
<td>32.7 (±23.15)</td>
<td>32.5(±15.7)</td>
<td>28.7 (±28.8)</td>
</tr>
<tr>
<td>3rd yr</td>
<td>35.3 (±20.5)</td>
<td>28.5(±16.3)</td>
<td>29.4(±12.3)</td>
<td>26.2 (±21.9)</td>
</tr>
</tbody>
</table>

(Where the normal levels of the above enzymes are:

AST*: the ULN range is ≤37 U/L for males and ≤31 for females.

ALT**: the upper limit of normal (ULN) range is ≤40 U/L for males and ≤31 U/L for females.

GGT***: the ULN range is ≤51 U/L for males and ≤33 U/L for females.

ALP****: the ULN range is ≤117 U/L for males and females adults.)
Table (3.6): Sensitivity, Specificity, Likelihood ratio and predictive value of AFP as a screening test for HCC.

<table>
<thead>
<tr>
<th>Test outcome</th>
<th>Conditions Positive (HCC)</th>
<th>Conditions Negative (no HCC)</th>
<th>Positive Predictive value = TP/(TP+FP) =94%</th>
<th>Negative Predictive Value = TN/(FN+TN) =84.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AFP as a screening test outcome</strong></td>
<td>Positive</td>
<td>False Positive</td>
<td>True Positive (TP)=16</td>
<td>False Positive (FP)=1</td>
</tr>
<tr>
<td>Test outcome</td>
<td>False Negative</td>
<td>True Negative</td>
<td>False Negative (FN)=9</td>
<td>True Negative (TN)= 49</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Sensitivity =TP/(TP+FN)</td>
<td>Specificity</td>
<td>Specificity =TN/(FP+TN)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>=64%</td>
<td></td>
<td>=98%</td>
<td></td>
</tr>
<tr>
<td><strong>Likelihood ratio (LR)</strong></td>
<td>Positive +LR = Sensitivity/(1-Specificity)=32</td>
<td>Negative –LR= (1-Sensitivity)/Specificity =0.37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Conc. of AFP (ng /ml)

Figure (3.1): Standard curve for AFP (Standard run with optical density reading at 450nm shown in the Y-axis against AFP concentrations shown in the X-axis)
Figure (3.2): Distribution of patients with liver cirrhosis according to sex
**Age of patients (years)**

*Figure (3.3): Distribution of patients with liver cirrhosis according to Age Groups*
Figure (3.4): Distribution of patients with LC and HCC according to causes
Figure (3.5): Cumulative hazard of HCC by the clinical cut-off values of AST enzyme. (Log rank $P=0.741$, HR=$0.669$ (95% CI = $0.061$-$7.381$), OR: $1.527$ (95% CI=$0.134$-$17.4$)
Figure (3.6): Cumulative hazard of HCC by the clinical cut-off values of ALT enzyme. (Log rank $P=0.198$, HR: $0.235$ (95% CI =0.021-2.593), OR: $4.467$ (95% CI=0.39-51.183).
Figure (3.7): Cumulative hazard of HCC by the clinical cut-off values of ALP enzyme. (Log rank P=0.039, HR: 0.121 (95% CI= 0.011-1.388), Odd ratio: 9.2 (95% CI=0.709-119.4).
Figure (3.8): Cumulative hazard of HCC by the clinical cut-off values of GGT enzyme. (Log rank $P=0.107$, HR=0.175 (95% CI=0.016-1.93), OR: 6.08 (95% CI=0.528-70.09)
Figure (3.9): Cumulative hazard of HCC by the clinical cut-off values of AFP.

(Log rank P=0.000, relative risk: 1.214 (95%CI= 0.974-1.513), HR: 1.00 (95% CI=0.00-3.155).
Figure (3.10): AFPmRNA detected in blood of patients with HCC. (PCR product (101 bp) of AFPcDNA using nested RT-PCR).
Figure (3.11): Distribution of patients with HCC according to Sex
Figure (3.12): Distribution of patients with HCC according to Age Groups
Chapter Four

DISCUSSION

In this study, patients with liver cirrhosis were studied and followed up. The mean age of the patients with LC was significantly lower than patients with HCC, but it was 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.\(^{71,72}\)

Hemoglobin levels were significantly lower in 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 consequently anemia due to chronic disease.\(^{135}\)

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 results who found that ALP was associated with increased risk for HCC in patients with LC.\(^{132}\) According to the previous study GGT enzyme also had a significant relation with increased HCC risk and also a significant association between GGT and overall cancer incidence.\(^{137}\) but this was not the case in our study which was in concordance with results of Wannamethee G. et al who found no significant association between GGT and cancer. It is on note that that GGT levels could be affected by factors like dietary, environmental pollutants and xenobiotic.\(^{137}\)
LC in this study group was caused mainly by HBV, in agreement with other studies performed in Sudan which indicated that HBV is of high seroprevalence\textsuperscript{16}.

HCV infection was found to be the fourth cause of liver cirrhosis which reflects the low seroprevalence of this virus among our patients. This finding 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\%),\textsuperscript{29} compared to high seroprevalence among end stage renal disease patients on hemodialysis\textsuperscript{32}.

Alcohol consumption was found to be the second cause of liver cirrhosis among the group with 28\% of patients acknowledging. This in contrast to studies in USA where alcohol is the most common cause of liver cirrhosis\textsuperscript{109}.

A significant relation was found between the level of AFP in patients with liver cirrhosis and increased HCC risk during follow up which was also confirmed by other studies\textsuperscript{112}.

During follow up, HCC nodules were detected by U/S and AFP in 3 patients only (3\%) and this could be due to many factors like the small number of patients included in the study and the low quality of U/S machines available in hospitals. Also the need for more experienced sonographers to detect small nodules less than 2cm is another factor. This is in agreement with the a study carried by Bruix et al who suggested that the time from an undetectable lesion to 2 cm is about 4–12 months; so surveillance for HCC needs specific training to enable efficient use of the diagnostic capabilities of the modern equipments\textsuperscript{103}. This may not be the case in Sudan where the patients are not
compliant with the follow up and usually present with large nodules. In addition, the fact that about 35% of HCCs < 2 cm could be missed by U/S. In comparison, in another study the detection of early small HCC nodules was found to be 73.2%. In other studies, the follow up of patients with LC by AFP level and U/S resulted in detection of HCC tumor in 8.2%, 19.5%, 20.7% and 26.85% of the patients with liver cirrhosis. In our study we detected HCC in 3% of the patients with LC.

AFP-mRNA was not detected in any of our patients with liver cirrhosis who have no HCC which is similar to Liu Y et al, results. However, it was detected in the three patients who developed HCC during follow up. This is comparable to other studies where AFP-mRNA was detected in 13.3%, 13.6%, 43.8% patients with liver cirrhosis respectively. Generally AFP-mRNA was markedly expressed in HCC patients compared to patients without HCC.

Sensitivity and specificity of AFP for detection of HCC in this study was 64% and 98% respectively which is in concordance with other studies with AFP sensitivities ranging from 41 to 69% and specificities between 75 and 94%.

When both AFP and U/S were accompanied the sensitivity and specificity increased to 84% and 100% respectively compared to a more recent study which evaluated the effectiveness of surveillance for HCC with U/S and AFP concluding that the combination of the two tests increased the sensitivity to 90.2%, with a small decrease in specificity (83.3%), in another study Sensitivity significantly improved to 90%, with minimal loss in specificity (83%) when the tests were used in combination.
Close relationship between the expression of AFP-mRNA and HCC occurrence, development, metastasis and prognosis. Using nested RT-PCR, AFP-mRNA was detected in 24% of our patients with HCC. These results are similar to previous studies where AFP-mRNA was detected in 25% of HCC cases. This is low compared to other results where the detection of AFP-mRNA was 30%, 54.5%, 53.8%, 59.5%, 48.1%, 73.3%, 72.1% respectively. The sensitivity of AFP-mRNA increased to 66.67% in AFP level; higher than 200ng/dl and this represents the only significant relation between the two biomarkers which is in line with another study. This differs from results which concluded that a significant relation was found between AFP-mRNA and AFP level (above 20ng/ml) in addition to other factors.

Most cases of HCC in whom AFP-mRNA was detected were HBV (+ve) (66.7%) compared to another study which indicated that no relation was found between AFP-mRNA and hepatitis B infection. This probably due to the high seroprevalence of HBV in Sudan.

**CONCLUSIONS**

Surveillance of patients with liver cirrhosis by ultrasound, AFP and AFP-mRNA assays in addition to routine liver biochemical values is the most effective strategy for early detection of HCC nodules.

One of the most significant findings to emerge from this study is that the combination of AFP and ultrasound is the most effective strategy for early detection of HCC nodules in Sudanese patients with liver cirrhosis. This is aided by alkaline phosphatase. AFP-mRNA has the lowest sensitivity though; high sensitivity was found by many researches outside Sudan. More future
studies are recommended for evaluation of this molecular marker in Sudanese patients.

The present study makes noteworthy contributions to the research on liver cirrhosis and HCC in Sudan and its most predisposing risk factors. It serves as a base for future studies on the effectiveness of the HCC screening programs and the most suitable predictive biomarkers.

A number of important limitations need to be considered in such types of studies mainly the patients’ incompliance, lack of adherence, the low quality of ultrasound machines available in hospitals and the need for expert sonographers to detect small hepatic nodules.

Large randomized controlled trials could provide more information regarding screening of Sudanese patients with LC by the above predictive tools and its cost-effectiveness. A major challenge ahead is the funding of such programs as most of patients are poors.

**Recommendations**

The early detection of hepatocellular carcinoma nodules on top of liver cirrhosis depends largely on the availability of high quality ultrasound machines and expert sonographers. So the followings are recommended:

1. Provision of high quality ultrasound machines is essential for any surveillance program for early detection of HCC in patients with liver cirrhosis.
2. In order to be effective, a surveillance program for hepatocellular carcinoma must involve training of sonographers for early detection of hepatic nodules on top liver cirrhosis.
3. Motivation of patients to adhere to the scheduled program is of utmost importance for successful future programs.
4. Large randomized controlled trials are essential to evaluate the effectiveness of screening by AFP-mRNA in Sudanese patients and other sensitive predictive biomarkers.

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Comprehensive analysis of common serum liver enzymes as prospective


APPENDIX

Questionnaire

Patient Name-------------------------
Age-----------------
Sex---------------------
Residence---------
Origin---------------------
Occupation-----------
Ethnic group---------
Date of admission-------
Address of relatives in Khartoum-----------------------------------------------
Present complaints ---------------------------------

SOCIAL HISTORY:
Marital status:
Single(    )  married (    )  divorced (    )
Alcohol intake:
Now (    )  formerly (    )
Type of alcohol taken-----------------------
Amount per day----------------------
Duration-------------------------------

Drugs (  )

Type------------------------

Past history:
Jaundice (  )  blood transfusion (  )

Medical treatment:

Disease-------------------------------

Duration--------------------------------------------------------------------------------

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Surgical treatment-------------------------------

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Medical examination:

Weight-----------------------------height------------------jaundice(  )

Cardiovascular system:

Pallor----------------

Blood pressure:---------

Pulse-------------------

Respiratory system:-------------------------------------------------------------------

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Abdominal examination:

Abdominal veins---------

Abdomen : hepatomegaly (  )
Liver size (in cm)
Consistency: soft ( ) firm( ) hard ( )
Surface----------------------------------
Lobe predominantly involved-----------------------------
Splenomegaly ( )
Stigma of cirrhosis ( ) specify----------------------
Venous hum ( ) bruit ( )
Ascites ( )

Laboratory investigations:
Hemoglobin gldl ( %) ESR
TWBCS lymph % mon % bas %
Urine general
----------------------
----------------------
Liver function tests
Total protein gldl
Albumin gldl
Bilirubin mgldl
INR
AST ALT Alkaline phosphatase GGT
HBsAg Anti-HCV antibodies
Serum Alpha fetoprotein

Alpha fetoprotein mRNA

Ultrasound

Diagnosis:

Plan of treatment

Follow up:

Remarks: