MOLECULAR APPROACH FOR EARLY DIAGNOSIS OF HEPATOCELLULAR CARCINOMA IN EGYPTIAN PATIENTS BY ALPHA FETOPROTEIN (AFP) AND VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

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ABSTRACT
The primary marker for Hepatocellular Carcinoma (HCC) is AFP. Generally AFP shows acceptable sensitivity, however AFP is not secreted in all cases of HCC and may be normal as many as 40% of patients with early HCC. This work aims to study and to evaluate biochemical diagnosis of HCC by comparing liver functions including Alpha fetoprotein (AFP) and its rule as a tumor marker or HCC in different groups that studied (Cases of HCC, cases at high risk, chronic hepatitis and cirrhosis against control group) and evaluated the value of measuring serum Alpha fetoprotein (AFP) and Vascular Endothelial Growth Factor (VEGF) for early diagnosis of HCC in HCC cases and in high risk groups. This study was conducted on three groups, group I cases of (HCC) (35 patients), group II cases at high risk to develop HCC (Cirrhosis, HBs Ag positivity and Anti-HCV) (35 patients), selected from the National Liver Institute, Menoufiya University and Group III 30 healthy control persons.

INTRODUCTION
Hepatocellular carcinoma (HCC) is the most common type of malignancy and is the most common cause of cancer death worldwide. Although the major etiological risk factors for HCC have been unraveled, it is clearly linked to infection with hepatitis virus (1). HCC is the fourth most common cancer worldwide and the third most common cause of cancer related death (2). Hepatocellular Carcinoma is more common in males than females with the ratio 4:1. This ratio varies widely according to the geographic distribution and may reach up to 8:1. In Egypt, liver cancer is the third most common cancer site for males after bladder and lung cancers and constitutes 5.5% of all cancers. For females, it is the fourth after breast, cervix and bladder cancers and constitutes 3.7% of all cancers (3).

The percentage of cases of HCC attributable to HBV worldwide is 52.3% and is higher in Asia where the sero-prevalence of HBsAg in the population is high. However, the vaccination campaign against the virus in some eastern countries has tended to lower the incidence of new cases of HCC (4). HCC is considered the fifth most common solid tumor in the world and accounts for about 500,000 deaths each year and the incidence of HCC is not uniform across the world but varies according to the prevalence of the underlying disease (5).

Hepatitis C may be more important than hepatitis B in the aetiology of HCC. Around 170 million people are infected with hepatitis C virus (6). Chronic hepatitis C is a major health problem world-wide, with approximately 200 million affected individuals and a significant rate of progression to end-stage cirrhosis and HCC (7). Patients with liver cirrhosis are at significant risk of hepatocellular carcinoma (HCC) that may
develop as well defined nodular lesions as more aggressive infiltrating tumours (8).

Cirrhosis may be a premalignant condition irrespective of the aetiology (9). There is a high prevalence of cirrhosis in patients with HCC (between 60-90%). There is an increased risk of patients with cirrhosis to develop HCC. In one series of 1073 HCCs, 658 (61.3%) also showed cirrhosis [9].

The primary marker for HCC is AFP, generally AFP shows acceptable sensitivity. However AFP is not secreted in all cases of HCC and may be normal as many as 40 % of patients with early HCC (10). It was estimated that HCC totally represents not less than 2.6% of body cancers and 5% of cirrhotic patients will develop HCC [11].

This work aims to evaluate the value of measuring serum Alpha fetoprotein (AFP) and Vascular Endothelial Growth Factor (VEGF) for early diagnosis of Hepatocellular Carcinoma (HCC) in HCC cases and in high risk groups as liver cirrhosis and chronic hepatitis.

PATIENTS AND METHODS

The present study was carried on 70 patients, 35 subjects suffering from hepatocellular carcinoma (HCC), 35 subjects suffering from liver cirrhosis and 30 persons of the same socio-economic group with matched age and sex used as healthy control individuals. All studied subjects were subjected to the following:

1. Thorough history taking.
2. Full clinical examination.
3. Abdominal ultrasonography.
4. Liver biopsy.
5. Laboratory investigations (liver function tests and total and direct bilirubin).

Quantitative determination of direct bilirubin (D.Bil) and total bilirubin (T. Bil) in serum was done using BIL-T kit manufactured by Roche Diagnostic, Germany (12).

Aspartate aminotransferase (AST), alanine aminotransferase (ALT); through colorimetric determination of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) activity, using transaminases kit provided by bio-Merieuxsa France (13). Alkaline phosphatase through colorimetric determination of alkaline phos-phasate (ALP) activity using Phos-phasate alkaline kit, supplied by bio Merieuxsa, France (Belfield arid Goldberg) (14).

Total Protein through colorimetric determination of total Protein (TP) using kit provided by Biocon, Germany (15). Albumin was determined through colorimetric test for albumin (ALB) concentration in serum sample using Albumin liquicolor kit, supplied by Human Gesellschatt for Biochemica and Diagnostica GmbH, Germany (16).

Prothrombin time was analyzed by coagulation process triggered by incubation of plasma with the optimal amount of thromboblastin and calcium and the time of formation of fibrin clot is measured (17) using the thromborel S kit, Germany.

VIRAL MARKERS: Hepatitis B Surface Antigen (HBsAg) using Sorin Biomedica Co. kits, Italy (18). The method is a direct, non-competitive sandwich assay based on the ELISA technique (Enzyme-Linked Immunosorbent Assay).

Hepatitis C virus Antibody (HCV-Ab) using Murex anti-HCV (version 4.0) provided by Abbott Diagnostic Division, Murex Biotech S.A., Republic of South Africa. Murex anti-HCV is an enzyme immunoassay for the detection of antibodies to HCV in human serum (19).

Quantitative determination of serum
alpha-feto protein (AFP) was done by Immunoenzymatic Assay Kit (20). The method used for quantitative AFP determination is a "one step" immune-enzymatic assay (IEMA) based on formation of a "sandwich" between the analyte to be detected and two specific monoclonal antibodies directed to different epitopes on the AFP molecule.

Quantitative determination of serum vascular endothelial growth factor (VEGF) was through the Enzyme Immuno-assay for the detection of total Human Vascular Endothelial Growth Factor from cytimmune (USA). Human VEGF is a competitive enzyme immunoassay (EIA), which measures the natural and recombinant forms of the cytokine vascular endothelial growth factor (VEGF) (21).

RESULTS

Regarding age and gender, there is no statistical significant difference between different groups according to age and gender. HBsAg in the cirrhosis group was 4 positive cases (11.4%) while there were 6 positive cases (17.1%) in the HCC group and the entire control group was negative to the HBsAg. There was no significant difference between different groups according to the distribution of the results of HBsAg. The HCVAb in the cirrhosis group was 22 positive cases (62.9%) while there were 21 positive cases (60.0%) in the HCC group and the entire control group was negative to the HCVAb. There was no significant difference between different groups according to the distribution of the results of HCVAb.

Regarding difference in the serum albumin among the studied groups, the mean ± SD of serum albumin in the cirrhosis group was 1.8± 0.4 with range while the mean ± SD in the (HCC) group was 2.2±0.7 and the mean ± SD in the control group was 4.1± 4.0. There was a significant difference between different groups according to serum albumin.

Regarding differences in the serum total protein among the studied groups, the mean ± SD of serum total protein in the cirrhosis group was 6.2±0.9 while the mean ± SD in the (HCC) group was 6.5±1.2 and the mean ± SD in the control group was 7.0±0.7 with range 6.5-7.8. There was a significant difference between different groups according to serum total protein. The significant difference was between control group versus cirrhosis group and between the control groups versus (HCC) group.

Regarding differences in the serum total bilirubin among the studied groups, the median of serum total bilirubin in the cirrhosis group was 5.20 with mean rank 66.9 while the median in the (HCC) group was 3.10 with mean rank 61.6 and the median in the control group was 0.80 with range mean rank 17.1. There was a significant difference between different groups according to serum total bilirubin. Regarding difference in the serum direct bilirubin among the studied groups, the median of serum direct bilirubin in the cirrhosis group was 2.20 with mean rank 66.9 while the median in the (HCC) group was 1.10 with mean rank 61.1 and the median in the control group was 0.15 with range mean rank 16.6. There was a significant difference between different groups according to serum direct bilirubin.

Regarding difference in the serum ALT among the studied groups, the mean ± SD of serum ALT in the cirrhosis group was 24.5±12.9 while the mean ± SD in the (HCC) group was 30.7±17.1 and the mean ± SD in the control group was 18.6±7.3.
There was a significant difference between different groups according to serum ALT. The significant difference was between cirrhosis group versus HCC group and between the control groups versus HCC group.

Regarding difference in the serum AST among the studied groups, the mean ± SD of serum AST in the cirrhosis group was 86.7±38.4 while the mean ± SD in the (HCC) group was 1004±43.8 and the mean ± SD in the control group was 23.4±7. There was a significant difference between different groups according to serum AST. The significant difference was between cirrhosis group versus control group and between the HCC groups versus control group.

Regarding difference in the serum alkaline phosphate among the studied groups, the mean ± SD of serum alkaline phosphatase in the cirrhosis group was 98.3±57.2 while the mean ± SD in the (HCC) group was 133.7±50.4 with and the mean ± SD in the control group was 62.0±14.9. There was a significant difference between different groups according to serum alkaline phosphatase. The significant difference between all groups alternatively.

Regarding difference in the prothrombintime (sec.) among the studied groups, the mean ± SD of prothrombin time (sec.) in the cirrhosis group was 20.5±4.7 with while the mean ± SD in the (HCC) group was 17.4±4.1 and the mean ± SD in the control group was 12.0±0.0. There was a significant difference between different groups according to prothrombintime (sec). The significant difference between all groups alternatively,

Regarding difference in the serum AFP among the studied groups, the median of serum AFP in the cirrhosis group was 6.9 with mean rank 44.8 while the median in the (HCC) group was 781.9 with mean rank 78.5 and the median in the control group was 3.9 with range mean rank 23.8. There was a significant difference between different groups according to serum AFP.

Regarding difference in the vascular endothelial growth factor (VEGF) among the studied groups, the mean ± SD of serum VEGF in the cirrhosis group was 4.7±1.4 with while the mean ± SD in the (HCC) group was 244.3±111.1 and the mean ± SD in the control group was 4.0±1.1. There was a significant difference between different groups according to serum VEGF. The significant difference was between HCC group versus cirrhosis group and between the HCC groups versus control group.

Regarding correlation between AFP and VEGF, there was a significant difference between the AFP level and VEGF level in the HCC group while there was no significant difference between both of them in the cirrhosis group. Also there was a significant difference between both of them in the control group.

**Figure-1:** Receiver Operating Characteristic (ROC) curve analysis indicates sensitivity and specificity of AFP in prediction of occurrence of hepatocellular carcinoma (HCC).
ROC curve shows that AFP level at cutoff point of 312ng/ml has sensitivity of 76.5% and specificity of 96.9% in prediction of occurrence of hepatocellular carcinoma.

Figure-2: ROC curve shows that AFP level of 312ng/ml is a cutoff point beyond which the occurrence of HCC dramatically increased. The ROC curve that indicates sensitivity and specificity of VEGF in prediction of occurrence of hepatocellular carcinoma (HCC).

A ROC curve show that VEGF level at cutoff point of 40.5ng/ml has sensitivity of 94.3% and specificity of 100% in prediction of occurrence of hepatocellular carcinoma. ROC curve shows that VEGF level of 40.5ng/ml is a cutoff point beyond which the occurrence of HCC dramatically increased.

DISCUSSION
As regards age distribution among HCC patients, it was in agreement with El-Zayadi et al, 2005(22) who found that the most predominant age group is 40–59 years and this shift to younger age group may be attributed to the emergence of HCV infection, as well as the acquisition of both B and C virus infection at younger age but this result was statistically insignificant, owing to the small sample size of this study.

Regarding gender distribution, HCC occurs in men more than in women. That is in agreement with Bosch et al, 2004 (23) who found that men were higher risk than women for HCC with a ratio ranges between 1.3 and 3.6 times higher in men in different parts of the world. The present study documented that HCC is more prevalent in men than in women and that the male gender is an independent risk factor for HCC but this result was statistically insignificant, owing to the small sample size of this study. This may be at least in part explained by the differences in exposure to risk factors, however, sex hormones and other X-linked genetic factors may be also important (24) speculated that estrogens and androgens could modulate hepatocarcinogenesis and explain the higher incidence of HCC in men.

Regarding some indicators of the synthetic function of the liver (S. albumin and total protein), in this study there was a significant difference between both S. albumin and total protein and the three groups. In relation to the results of serum albumin, there was a significant decrease in its level in cirrhotic group and in HCC group than control group. In this study, there was a significant decrease in the level of serum total proteins in the groups of cirrhosis, and HCC, in comparison with the control group.

Regarding hepatobiliary functions of the liver (total bilirubin and direct bilirubin), there was a significant difference between the total and the direct bilirubin and the three studied groups. It was found that serum total bilirubin was significantly increased in cirrhotic group and in HCC group than in control group. There was also a significant increase in the direct
bilirubin in this study in the cirrhosis and the HCC group in relation to the control group.

Regarding prothrombin time (PT), the results of this study show a significant difference between the prothrombin time and the three studied groups. The significant difference was between control and the other two groups (25) reported that in liver disease, PT may be abnormal because of multiple deficiencies of coagulation factors synthesized in the liver. Thus, a prolonged PT in conjunction with other evidences of liver disease is an important finding and a measure of disease severity.

Regarding the liver enzymes, the results of this study show significant increase of the serum transaminases ALT and AST in their levels in the cirrhotic and the HCC groups in relation to the control group. In this study we found that the distribution of the results of HBsAg and HCVAb among groups of the studied population show no significant difference as the regarding the small sample size in the present study.

As there was a 62.9% of the cirrhotic group proved to be HCV Ab positive, while 60% of the HCC group proved to be HCV Ab positive. Also we found that 11.4% of the cirrhotic group proved to be HBsAg positive, while 17.1% of the HCC group was HBsAg positive.

The study also revealed that a significant higher level of AFP in both cirrhosis and HCC groups when compared to the control group and in HCC group when compared to the cirrhotic group. These results agree with those reported by Johnson 2001 (26), who found that serum AFP is increased in cases of cirrhosis as well as other non-malignant conditions such as viral hepatitis and pregnancy particularly if the pregnancy is complicated by a spinal cord defect or other abnormality.

So in our study regarding the ROC curve that indicates sensitivity and specificity of AFP in prediction of occurrence of hepatocellular carcinoma we found that ROC curve shows that AFP level at cutoff point of 312ng/ml has sensitivity of 76.5% and specificity of 96.9% in prediction of occurrence of hepatocellular carcinoma.

ROC curve shows that AFP level of 312ng/ml is a cutoff point beyond which the occurrence of HCC dramatically increased. Also, in our study, serum VEGF was higher in HCC group than control group and cirrhotic patients. There is a significant difference between different groups except there is no significant difference between controls and cirrhotic patients without HCC.

The VEGF levels were not significantly different between patients with liver cirrhosis without HCC and normal controls. So in our study regarding the Roc curve that indicates sensitivity and specificity of VEGF in prediction of occurrence of hepatocellular carcinoma we found that:

ROC curve shows that VEGF level at cutoff point of 40.5ng/ml has sensitivity of 94.3% and specificity of 100.0% in prediction of occurrence of hepatocellular carcinoma.ROC curve shows that VEGF level of 40.5ng/ml is a cutoff point beyond which the occurrence of HCC dramatically increased.
REFERENCES


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