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P53 and endoglin, two biomarkers for predicting HBV and hepatocellular carcinoma in Egyptian patients

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Abstract

Background: liver biopsy is considered the golden standard for the investigation of fibrosis and hepatocellular carcinoma (HCC). However, liver biopsy is costly and may lead to medical complications in some cases. Therefore, the search for non-invasive biomarkers is a good alternative.

Aim: the present study aims to analyse the interactive role of serum alpha fetoprotein (AFP), soluble endoglin and p53 in diagnosis of patients with chronic hepatitis B virus (HBV), and early stage HCC.

Patients and methods: This study included 56 patients, divided into three groups. Group I (control): Composed of 18 healthy controls. Group II (HBV): included 13 chronic HBV. Group III (HCC): included 25 newly diagnosed, stage II HCC. Viral markers, liver function tests, blood sugar level, kidney function tests and AFP were assessed using standard methods. Serum levels of p53 and soluble endoglin were determined using ELISA kits.

Results: HBV Group showed normal liver functions except for higher total protein and albumin mean levels, whereas HCC group revealed significantly higher ALT, AST, ALP, total and direct bilirubin. The mean AFP serum levels were significantly increased in HBV and HCC groups in relation to the control group in an ascending order. The mean value of soluble endoglin was significantly altered in HBV and HCC group compared to that of control group. Only HCC group showed a significant increases in p53 level compared to control group.

Conclusion: the results showed that p53 and endoglin are potential biomarkers that can help in the diagnosis of hepatocellular carcinoma when used in addition to AFP.

Key words
HCC, HBV, p53, endoglin

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer related death in the world and the fifth most frequently diagnosed cancer worldwide [1, 2]. The prognosis of HCC is subject to a lot of factors, among those are chronic persistent infection with hepatitis B (HBV) and C (HCV) viruses [3, 4]. About 80% of HBV-infected individuals fail to eliminate the virus, and hence progress to chronic infection. Continuous inflammation, fibrosis and subsequent progression to cirrhosis are thought to lead to chromosomal damage and possible initiation of hepatic carcinogenesis [5, 6].

Previous studies showed that the transient transfection of HBV into hepatic cell lines enhanced p53 expression (human tumor suppressor gene) and played a role in development of HCC [7]. However, viral-related HCC often takes place following many years of viral infection, when the disruption of the balance between proliferation of cells and apoptosis in viral infected hepatic cells occurs [7].

Endoglin is a membrane glycoprotein that acts as a co-receptor for TGF-β1. It is involved in the development of cardiovascular system and vascular remodeling, and is strongly expressed in tumor tissues vasculature. Therefore, it plays a role in tumor progression, survival and metastasis [8]. During fibrosis process, its expression was reported to be increased in kidney and liver tissues, which connects its serum level to bad prognosis [9].

Alpha fetoprotein (AFP) is reported to be elevated in HCC [10, 11]. Unfortunately, some researchers reported a normal serum level of AFP in 40% of patients with early stage HCC, which decreases its diagnostic value in such cases [12].

Nowadays, there is an urgent need to find additional sensitive serum markers to identify patients at risk of progression into cirrhosis and those with an early stage HCC. However, based on

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the fact that HCC is a highly heterogenous disease, using a combination of highly specific markers should provide a better tool for HCC diagnosis [13].

So, the present study aims to analyse the significance of serum soluble endoglin and p53 in patients with chronic HBV and early stage HCC. The present study also aims to investigate whether these parameters could identify and discriminate patients with chronic HBV and those with an early stage HCC.

2. Patients and methods

2.1 Design of the experiments

The current study included a total of 56 human subjects (50 males and 6 females) that were selected from the attendants of the outpatient clinic of the gastroenterology department, Minia university Hospital, Minia, Egypt. The protocol used was approved by the ethics review board of the biochemistry department, faculty of pharmacy, Minia University and in accordance with the ethical standards of Helsinki declaration (JAMA 2000; 284:3043-3049) and all patients provided a written consent to participate in the study.

The study subjects were subdivided into 3 groups as follows:

Group I (control): included 18 completely normal healthy controls.

Group II (HBV): included 13 patients with clinically and serologically proven chronic HBV infection.

Group III (HCC): included 25 newly diagnosed, clinically, radiologically- and histopathologically-proven (data not shown), stage II HCC. Among them 68 % had past history of HBV.

The age of the participants ranged from 44 to 77 years. Included individuals in HBV and HCC groups had no previous treatment for 6 months. The following investigations were done for each participant: complete and detailed medical history with special emphasis on past history, complete physical examinations (general, chest, heart and abdominal), ultrasound evaluation, CT or MRI scan. In addition, routine laboratory investigations including viral markers, liver function tests, blood sugar, kidney functions and AFP using standard methods, serum levels of p53 protein and soluble endoglin were determined.

2.2. Sample collection

Eight milliliters of venous blood (Fasting morning samples) were collected from each participant on admission, in addition to liver biopsies for histopathological analysis. To investigate prothrombin time (PT), two milliliters of blood were placed in a tube containing Na-citrate (3.2%). The remaining blood was first allowed to clot for 15 minutes then centrifuged for 10 minutes at 5000x g and sera were separated and stored in aliquots at -20°C till analysis.

2.3. Laboratory analysis

-Analysis of liver functions (ALT, AST, albumin, total proteins, alkaline phosphatase (ALP) and γ- glutamyltransferase (GGT)) were done as routine laboratory tests using BM Hitachi 911 chemical analyzer according to manufactures instructions.

-Kidney functions (serum blood urea nitrogen (BUN), creatine and glucose) were done routinely using commercially available kits according to manufacturer's instruction.

-Viral markers (HBsAg, HBeAg, HBV-DNA and Anti–HBc) were estimated by ELISA kits (Biorad, USA) and Fast Real Time PCR system (CA, USA) (data not shown).

-Serum AFP levels were estimated using commercially available ELISA kits (Quantikine Human α- fetoprotein immunoassay, R&D systems,Minneapolis, MN).

-Prothrombin time (PT) & concentration were performed using Thromboril S® kit supplied by Dade Behring on Behnk Elektronik coagulator.

-The levels of serum p53 were measured using human p53 ELISA kit (Glory Science Comp. LTd., Del Rio, TX 78840,USA) as described before [14]. The kit uses a double antibody sandwich ELIZA to assay the level of human p53/tumor protein (p53) in serum samples.

-The level of serum soluble endoglin was determined using human endoglin ELISA kit supplied by WKEA MED Supplies Corp., Changchun 130012, China [15].

2.4. Statistical analysis

Using SPSS version 19, Chi-square test was used to compare between qualitative variables. Mann-Whitney test was used to compare between two quantitative variables in case of non-parametric data. Spearman correlation was done to measure correlation between quantitative variables. Medcalc was used to calculate sensitivity, specificity, positive and negative predictive values and ROC curves. P-value less than 0.05 were considered as statistically significant.

3. Results

3.1. Investigation of liver functions

Liver functions were assessed in all patient groups revealing that patients of HBV group had normal liver functions except for significantly higher PT, total- and direct bilirubin mean levels ($p<0.0001$, $p<0.0001$ and $p< 0.05$, respectively). HCC patients (HCC group) had significantly higher transaminases and ALP ($p<0.0001$), total and direct bilirubin ($p<0.003$ and $p<0.001$ respectively), in addition to significantly lower PT ($p<0.0001$) were observed in this group when compared to the control values **(Table 1)**.

3.2. Investigation of kidney functions

Comparing the kidney functions including blood urea nitrogen (BUN) and creatinine showed no significant differences among the different groups (data not shown).

3.3. Estimation of serum levels of AFP, p53 and soluble endoglin

The mean values of serum AFP, p53 as well as soluble endoglin showed a highly significant increase in HCC group compared to

Table 1: Levels of liver functions' parameters in sera of the different test groups

	Group I Control (n= 18)	Group II HBV (n= 13)	Group III HCC (n= 25)
PT (g/L)	64.16 ± 8.35	80.10 ± 5.38***	51.34 ± 8.83$^{***,###}$
Albumin (g/L)	31.52 ± 7.71	49.49 ± 4.44***	32.29 ± 27.10$^{\#}$
AST (IU/L)	27.36 ± 19.57	31.81 ± 17.94	129.40 ± 74.92$^{***,###}$
ALT (IU/L)	31.92 ± 15.24	33.08 ± 19.03	113.92 ± 69.33$^{***,###}$
ALP (IU/L)	112.33 ± 38.91	85.15 ± 23.83*	316.73 ± 152.58$^{***,###}$
Total Bilirubin (mg/dl)	0.65 ± 0.41	0.68 ± 0.40	1.90 ± 1.64$^{**,##}$
Direct Bilirubin (mg/dl)	0.27 ± 0.20	0.25 ± 0.14	0.99 ± 0.88$^{**,##}$
Glucose (mmol/L)	5.69 ± 0.77	5.20 ± 0.88	5.08 ± 1.07*

PT: thrombin time; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase. *: P<0.05, **: P<0.003, ***: p<0.0001 compared to control

$^{\#}$: P<0.05, $^{\#\#}$: P<0.003, $^{\#\#\#}$: p<0.0001 compared to HBV, values are expressed as Mean ± SD

the healthy control as well as HBV group (p<0.001, p<0.002 and p<0.002 respectively, **Table 2 and Figures 1, 2 and 3)**. On the other hand, a significant increase in AFP and a significant reduction in soluble endoglin levels were detected in HBV group (p<0.002 and p<0.05 respectively, **Table 2 and Figures 1, 2 and 3)**, whereas no significant change in P53 level compared to control was observed in this test group (p>0.05). Comparing HCC group to HBV group showed a significant increase in AFP, p53 and endoglin (p<0.001, p<0.003, p<0.001 respectively).

Table 2: Serum alpha fetoprotein (AFP), p53 protein & endoglin levels in the serum of patient' groups compared to the controls

	Group I Control (n= 18)	Group II HBV (n= 13)	Group III HCC (n= 25)
AFP (ng/ml)	6.18± 6.11	17.69 ± 5.54***	197.58 ± 154.45$^{***,###}$
p53 (ng/ml)	0.52 ± 0.19	0.51 ± 0.35	1.19 ± 0.92$^{**,##}$
Endoglin (ng/ml)	5.49 ± 1.34	4.34 ± 1.08*	7.24 ± 1.71$^{**,###}$

AFP: alpha fetoprotein; *: P<0.05, **: P<0.002, ***: p<0.001 compared to control

#: P<0.05, ##: P<0.003, ###: p<0.001 compared to HBV, values are expressed as Mean ± SD

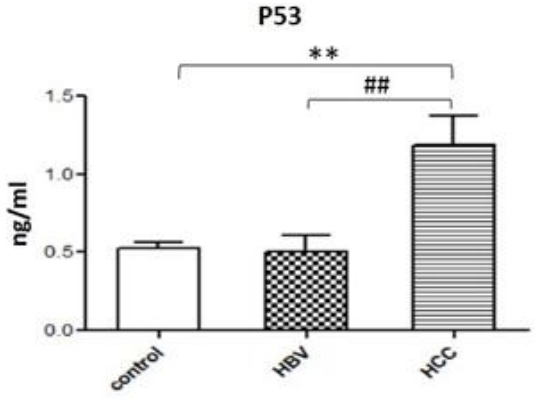

Figure 2: Serum P53 mean ±SD levels in the patient groups studied compared to the control group (*) or HBV group (#). P53 shows a significant increase in HCC group compared to healthy control as well as HBV group (**, ##, p<0.002 and p<0.003 respectively). No significant difference between P53 level in HBV and healthy control could be detected (p>0.05).

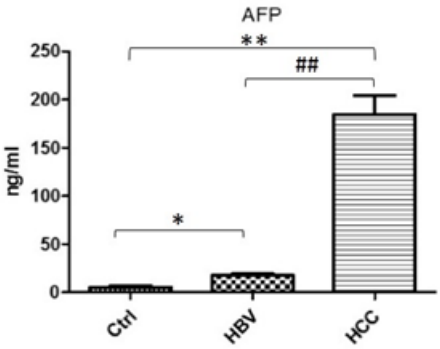

Figure 1: Serum AFP (alpha fetoprotein) mean ±SD levels in the patient groups studied compared to the control group (*) or HBV group (#). AFP expression is significantly higher in HCC group compared to healthy control (**,p<0.001) and HBV groups (##, p<0.001). HBV group also shows a significant upregualtion of AFP that is less prominent than HCC (*, p<0.002).

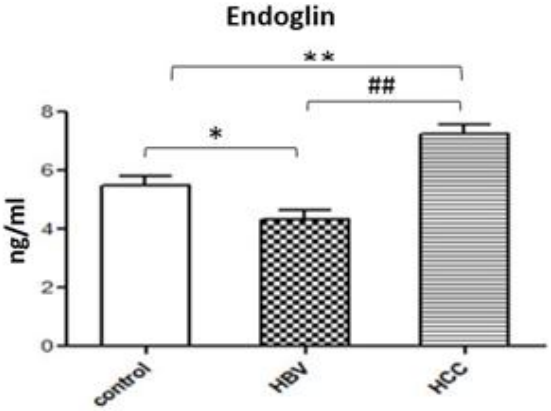

Figure 3: Serum soluble endoglin mean ±SD levels in the patient groups studied compared to the normal controls (*) or HBV group (#). HCC group showed a significant increase in serum endoglin compared to healthy control and HBV group as well (**, ##, p<0.002 and p<0.001 respectively). HBV group showed a significant reduction in serum endoglin compared to healthy control (*, p<0.05).

3.4. Correlations and cut-off values of the various biomarkers

Endoglin and P53 showed a significant positive correlation both in HCC and HBV group ($p<0.05$, $p<0.00007$ respectively), whereas a significant positive correlation between p53 and AFP was observed only in HCC group ($p<0.002$, **Table 3**). No positive correlation between AFP and endoglin was detected either in HBV or HCC groups.

Table 3: Spearman's correlation coefficients of serum p53, endoglin and AFP in HBV and HCC groups

Markers	HBV	HCC
P53/endoglin	r=0.7088 $p=7.3x10^{-5}$*	r=0.6239 p=0.0226*
AFP/endoglin	r=-0.0326 p=0.877	r=0.295 p=0.3278
P53/AFP	r=0.2381 p=0.2517	r=0.7939 p=0.0011*

R: Spearman's correlation coefficient, p: significance, P<0.05 is significant. *: significant

Tables 4 shows the sensitivities, specificities, positive and negative predictive values (PPV and NPV) and area under ROC curve (AUC) for these three variables (AFP, p53 and endoglin) in the HBV and HCC groups of patients respectively.

4. Discussion

In Egypt, there is a high prevalence of chronic liver diseases which progresses in many cases to hepatic fibrosis and cirrhosis that may consequently lead to hepatocellular carcinoma. So far, there is no established system for predicting the onset of HCC. Therefore, a way for identifying patients at a high risk of progression from liver fibrosis into cirrhosis is needed utilizing more sensitive and specific biomarkers that do not rely on biopsy alone [16, 17].

In our results, patients with chronic HBV showed normal liver functions (transaminases, ALP and bilirubin), which indicate a recovered non replicating form of the infection [18]. Chronic HBV usually leads to hepatocellular inflammatory damage which is a major risk factor for human HCC. It was shown previously that stress in adult liver can lead to epithelial-to-mesenchymal transition (EMT) [19]. Such transition causes some stromal cells to co-express epithelial markers (e.g. AFP, albumin and cytokeratins), as well as mesenchymal markers (e.g. osteopontin and collagen) [20], which may explain the observed increase in total protein and albumin in HCC group of patients compared to healthy control.

The patients of HCC group showed a condition of impaired liver functions in the form of decreased PT, increased albumin, bilirubin and ALP levels and increased serum AST & ALT enzyme activities. These effects can be regarded to hepatocellular damage with a higher degree of cholestasis [21].

In the present study, a significant increase in serum AFP was detected in both patient groups compared to healthy controls, with the highest value being detected in HCC group. These results are in line with previous studies reporting similar tendencies [22, 23].

Tumor suppressor p53 plays a central role in protecting the integrity of human genome and preventing cancer formation, causing the cancer onset to be critically determined by its integrity [24]. Wild type p53 is maintained at a very low concentration (undetectable) within the cell and exists mainly in an inactive latent form [25]. Viral infection as well as naturally-occurring or synthetic chemicals that accidentally contaminate food or water can cause the loss of p53 function via preventing its activation either by mutating p53 itself or mutating its downstream mediators [26-28]. These abnormal forms of p53 lack the ability to protect cells against genomic alteration, conferring to the uncontrolled growth of tumor cells [29].

On one hand, we did not notice an increase in the serum level of p53 in chronic HBV group. This could be explained on the basis that the recovered non-replicating hepatitis B virus may help the normal stability and activity of p53, which is consistent with previously reported results [7].

On the other hand, serum p53 protein levels were significantly

Table 4: The cutoff, sensitivity, specificity, PPV, NPV & area under the ROC curve for the three variables (AFP, P53 & endoglin) in HBV and HCC groups.

	HBV			HCC		
	Endoglin	**P53**	**AFP**	**Endoglin**	**P53**	**AFP**
Cut-off	> 5.85	> 0.635	> 58.7	> 5.85	> 0.635	> 58.7
Sensitivity	84	80.0	88.0	15.00	24	6
Specificity	50	66.7	98.0	50.00	66.6	85
PPV	57.5	66	97	8.31	17.8	4.8
NPV	79.5	80.5	91	66.01	74.4	18
AUC	0.784	0.812	0.984	0.767	0.594	0.833

PPV: positive predictive value; NPV: negative predictive value; AUC: area under the curve.

higher in case of HCC group when compared to the control group, in a similar tendency to that reported by other researchers [30, 31]. This elevation could be attributed to the continuous inflammatory state found in this group.

Endoglin is a co-receptor for TGF–β isoforms and is predominantly expressed in endothelia of vessels in inflamed, damaged, regenerating or proliferating tissues [32, 33]. The current study showed a significant decrease in serum soluble endoglin levels in the HBV group, while HCC group showed significantly higher values as compared to that of control group. These findings are in agreement with the results reported by *Yagmur et al.,* [34]**.** It is believed that endoglin can act as a proliferation marker that strongly correlates with proliferation in tumor endothelial cells.

The decreased levels of endoglin in HBV patients in the current study can be explained by the compensatory liver state in which endoglin expression is inhibited to enhance the normal ability of TGF–β to suppress proliferation and migration of endothelial cells. This could decrease focal adhesion & enhance apoptosis that is normally induced by the injured cells [35, 36].

The elevated levels of serum soluble endoglin detected in HCC patients can be attributed to the increased remodelling of ECM, proliferation and migration of endothelial cells and the angiogenetic conditions required for tumor progression [8, 34, 37].

In the present study, a significant positive correlation was observed between endoglin and AFP in the control group, which suggest that their combination can increase their sensitivities. However, this correlation was not noted in the patient groups which can be attributed to the relative small sample size.

Serum endoglin and p53 were positively correlated in HBV as well as HCC group. Hepatic cells in case of HCC as well as HBV express many cytokines including TGF–β, for which endoglin acts as a co-receptor. Endoglin expression by the inflamed liver cells, together with the produced cytokines, act as endogenous inducers of p53 expression, explaining the observed results.

The results presented in this study revealed that the sensitivity and specificity values of the different parameters are in agreement with those reported earlier [34], with the diagnostic sensitivities of 69%, 15% (in HBV) and 80%, 84% (in HCC) and specificity range of 50 and 66 % for p53 and endoglin respectively.

In conclusion, the results of the current study suggest that the combined use of AFP, p53 and endoglin provides a non-invasive, inexpensive alternative tool, which can successfully predict and differentiate between chronic HBV and HCC. It is noteworthy that the low number of subjects in the study represents a main limitation.

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Conflict of interest

no conflict of interest is to declare

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