RESEARCH COMMUNICATION

Comparison of Serum Survivin and Alpha Fetoprotein in Egyptian Patients with Hepatocellular Carcinoma Associated with Hepatitis C Viral Infection

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Abstract

> **Background:** Survivin is specific antiapoptotic gene product expressed in a variety of human neoplasmswhose overexpression might assist in early diagnosis and as a prognostic marker. **Objectives:** The aim was to evaluate the plasma levels of survivin and alpha fetoprotein in patients with chronic hepatitis C viral infection (HCV) with and without hepatocellular carcinoma (HCC). **Subjects:** 70 subjects were divided into: a control group (Group I) (20 healthy volunteers) and two patients groups: Group II, HCV group (20 patients); and Group III, HCC with HCV(30 patients). **Methods:** Thorough physical examination, ultrasonography of the abdomen, laboratory investigations (liver profile, anti-HCV antibodies, hepatitis B surface antigen, Alpha fetoprotein (chemiluminometry) and Survivin (ELISA)) were performed. **Results:** There was a significant increase in survivin level in HCV patients (Group II) when compared to the control group (p=0.039), along with a significant increase in AFP in Groups II and III when compared to Group I (P<0.001 for both). AFP also distinguished between the two HCV groups. The best generated cut off value for AFP was 10.9 ng/ml and for survivin 13.7 pg/ml. Serum survivin diagnostic sensitivity was 53.3%, diagnostic specificity 62.5% and efficiency 58.6%, in contrast to 100%, 92.5% and 95.7%, respectively, for AFP. **Conclusions:** While survivin showed significant increase in the HCV group, its diagnostic performance was lower and it proved to be less reliable as a tumor marker for HCC than did AFP.

Keywords: Apoptosis - survivin - hepatocellular carcinoma - alpha fetoprotein.

Introduction

Apoptosis is a tightly regulated process that involves complex signaling pathways and cascades of molecular events needed for proper development during embryonic and fetal growth where there is destruction and reconstitution of cellular structures. In adults, apoptosis is needed to maintain differentiated tissues, elimination of aged cells without disrupting cellular integrity. (Bokerawa et al., 2005; Ye et al., 2007).

The suppression of apoptosis is thought to contribute to carcinogenesis due to several mechanisms ; including unusually prolonging of cellular lifespan , facilitating the accumulation of gene mutations and permitting growth factor-independent cell survival (Mamori et al., 2007). In addition, since the host's immune system normally eliminates cancer cells by induction of apoptosis, inhibition of this process is critical for cancer survival. It has been shown that the "Inhibitors of Apoptosis Proteins''(IAP) are crucial regulators in the molecular mechanisms of apoptosis (Ito et al., 2000; Mamori et al., 2007).

Survivin, NIAP, c IAP1, c IAP 2, XIAP, Ts XIA , ML-IAP and Apollon are IAP family members ,and Survivin

being the smallest member (Fields et al., 2004; Bokerawa et al., 2005). Survivin also known as Birc 5, 142 amino acid , is a bifunctional protein that regulates cell division and suppresses apoptosis (Bokerawa et al., 2005; Duffy et al., 2007).

Survivin is unique among the IAP proteins not only because it exhibits cell cycle-regulated expression that peaks at mitosis, but also, because it is undetectable in the majority of normal adult tissues except for placenta, thymus, spleen, testes, colonic crypts, primitive hematopoietic cells, T lymphocytes, neutrophils and vascular endothelial cells. It may regulate their proliferation or survival (Altieri et al., 1999; Sarela et al., 2000; Chang et al., 2004; Fields et al., 2004; Fukuda et al., 2006; Duffy et al., 2007; Ye et al., 2007). Also survivin is essential for angiogenesis; a fundamental process in tissue regeneration (Chiou et al., 2007).

Survivin is abundantly expressed in transformed cells and a variety of human cancers (Kappler et al., 2001; Ikeguchi et al., 2002; Fields et al., 2004; Wang et al., 2004; Asanuma et al., 2005; Vetter et al., 2005). The potential utility of survivin overexpression in early diagnosis and as a prognostic marker of cancer is controvertible (Kappler

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et al., 2001).

It has also been shown that Survivin inhibits apoptosis in cells exposed to diverse apoptotic stimuli by associating with microtubules of the mitotic spindle and inhibiting caspase activity. However, the biological functions of survivin, other than its antiapoptotic effect, are not well understood in human cancers (Kobayashi et al., 2002).

Expression of Survivin may be of prognostic significance and therapeutic relevance in many cancers. The overexpression of Survivin in some cancer types can correlate positively with poor clinical prognosis ,increased rate of tumor recurrence and resistance to chemotherapy but does not correlate with overall survival (Kappler et al., 2001).

Anti-survivin therapies may potentiate chemotherapies that stimulates apoptosis by removal of the anti-apoptotic effect of Survivin and may also exert an anti-proliferative effect (Kappler et al., 2001).

Hepatocellular carcinoma (HCC), a major health problem worldwide, is one of the most common primary neoplasm of the liver and one of the most common solid tumors in the world (3). More than 500,000 new cases were diagnosed each year (Di Maio et al., 2008). HCC accounts for between 85% and 90% of primary liver cancer (Parkin, 2000). It is the fifth most common cancer worldwide and the third most common cause of cancer mortality (Bosch et al., 2005). In Egypt, HCC was reported to account for about 4.7% of chronic liver disease patients (El-Zayadi et al., 2001).

The most common condition associated with hepatocarcinogenesis is cirrhosis which develops 20 to 40 years of chronic liver disease. Major causes of cirrhosis in patients with HCC include hepatitis B, hepatitis C, alcoholic liver disease and possibly nonalcoholic steatohepatitis. (12) It has been reported that not only increased cellular proliferation but also, diminished cell death play important roles in hepatocarcinogenesis and tumor progression in HCC (Befeler et al., 2001).

Hepatocellular carcinoma develops from transformed hepatocytes during the course of chronic liver diseases . The activation of mitogenic oncogenes which is important for deregulated proliferation of hepatocytes, has been shown to enhance apoptosis. Therefore, resistance to apoptosis may provide a selective advantage factor for growth and progression of HCC. However, cellular mechanisms that inhibit apoptosis in HCC are not well understood (Ikeguchi et al., 2002) .

In HCC patients, Survivin expression has been frequently studied at cellular level and in tissues, but to our knowledge , little is known about its plasma level in such a disease (Ye et al., 2007; Hui et al., 2008).

The present work aimed at evaluating the plasma level of Survivin in Egyptian patients with HCC associated with hepatitis C infection and to compare it with its level in patients with chronic hepatitis C infection . The diagnostic performance of Survivin, as a possible tumor marker, will also be compared to that of serum Alpha fetoprotein.

Materials and Methods

This study was conducted on 70 subjects, after taking their consents, consequtively collected from the Medical and Oncology Departments of the Medical Research Institute, Alexandria Egypt divided into 2main groups: Control group (GroupI) included 20 apparently healthy volunteers and Patients groups(Group II and III) :
Group II : Hepatitis C viral infection group (20 patients) with HCV.
Group III:Hepatocellular carcinoma group (30 patients) with HCC on top of HCV infection.

Patients with rheumatoid arthritis, hepatitis B viral infection or other malignancies were not included .

To all the studied subjects thorough physical examination was done.All patients were classified using Child-Pugh score (Feldman et al., 2002) and ultrasonography of the abdomen were done. Laboratory investigations included: complete blood picture and plasma prothrombin activity (Lewis et al., 2001), fasting serum glucose (Trinder et al..1969), creatinine, protein ,albumin, total and direct bilirubin levels in addition to aminotransferases, alkaline phosphatase and gamma glutamyl transferase activities. Patients were screened for Rheumatoid factor, anti-HCV antibodies ,hepatitis B surface antigen, Schistosomal antibodies (Burtis et al., 2006). Alpha fetoprotein was performed by chemiluminometric method and Survivin by ELISA (Bishop et al., 2005).

Results

The control group included twenty apparently healthy volunteers (14 males and 6 females) their median age was 48.50 years (range=40-62years). The hepatitis C viral infection group (Group II) included twenty patients suffering from chronic HCV infection (12 males and 8 females) their median age was 50 years (range=40-67years). The hepatocellular carcinoma group (Group III) included thirty patients suffering from HCC associated with HCV infection (23 males and 7 females) their median age was 55 years (range=38-72years).

Ultrasonographic findings in the studied groups:

In HCV patients group (Group II): 9 out of 20 (45%) had enlarged liver with cirrhotic changes, 1 out of 20 (5%)
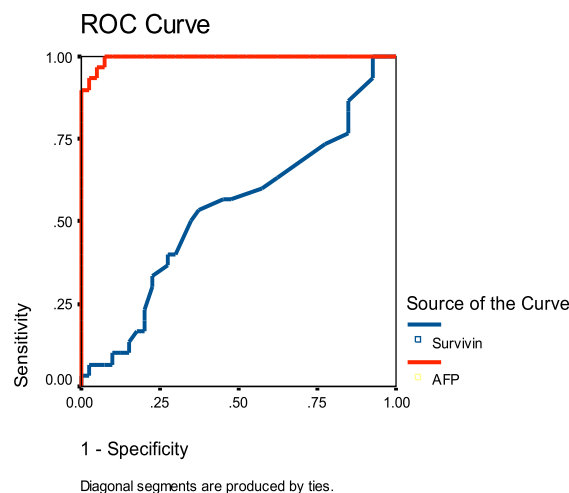
Figure 1. Receiver Operator Characteristic Curves of Survivin and Alpha Fetoprotein (AFP)

Table 1. Complete Blood Picture and Prothrombin Activity in the Studied Groups

Parameter	Group	Controls (GroupI) (n=20)	HCV Patients (Group II) (n=20)	HCC Patients (Group III) (n=30)
HB	Median	14	9.9*	12.2*•
(g/dl)	Min-Max	13-15.2	8.2-13.3	9.3-14.9
	P		0.000	0.000
	P₁			0.003
RBC'S	Median	4.9	3.6*	4*•
(cell/mm³)	Min-Max	4.7-5.3	2-4.1	2.7-4.9
X10⁶	P		0.000	0.000
	P₁			0.018
HCT	Median	40.05	28.1*	35.7*•
(%)	Min-Max	37.1-47.9	19.3-38.7	24.8-43.2
	P		0.000	0.000
	P₁			0.003
MCV	Median	80.4	83	88*
(fl)	Min-Max	72.7-90	60.8-105	70.3-107.2
	P		NS	0.003
	P₁			NS
MCH	Median	28.3	30*	30.8*
(pg)	Min-Max	26.5-30.2	13.7-42.7	25-34.6
	P		0.021	0.000
	P₁			NS
MCHC	Median	34.95	35.5	34.7
(g/dl)	Min-Max	31.7-37.7	31.6-42.8	23.2-41.6
	P		NS	NS
	P₁			NS
PLT	Median	299.5	162.5*	162*
(cell/mm³)	Min-Max	195-422	44-347	80-326
X10³	P		0.000	0.000
	P₁			NS
WBC'S	Median	5.4	6.25	5.5
(cell/mm³)	Min-Max	4.4-9.3	1.2-11.2	2-13.8
X10³	P		NS	NS
	P₁			NS
Basophils	Median	0	0	0
(%)	Min-Max	0-1	0-1	0-3
	P		NS	NS
	P₁			NS
Eosinophils	Median	1	2	3*
(%)	Min-Max	0-4	0-19	0-8
	P		NS	0.033
	P₁			NS
Staff neutrophils	Median	0	0.50	0
(%)	Min-Max	0-2	0-4	0-4
	P		NS	NS
	P₁			NS
Segmented neutrophils	Median	58	67.50*	63
(%)	Min-Max	49-67	40-94	48-88
	P		0.03	NS
	P₁			NS
	Median	38.5	25*	30*
Lymphocytes	Min-Max	32-45	4-54	10-40
(%)	P		0.000	0.000
	P₁			NS
Monocytes	Median	3	3	4
(%)	Min-Max	1-6	0-14	0-15
	P		NS	NS
	P₁			NS
Prothrombin activity	Median	96.45	55.8*	67.5*
(%)	Min-Max	78.1-100	43-100	25-100
	P		0.000	0.000
	P₁			NS

Min: minimum Max: maximum n: number of subjects. NS: No significant difference; *P: Statistical significance between control (group I) and both HCV patients (group II) and HCC patients (group III).; •P1: Statistical significance between HCC patients (group III) and HCV patients (group II).; P-value <0.05 was considered statistically significant.

Table 2. Serum Values of Fasting Serum Glucose (FSG), Creatinine(Cr), Total Protein (TP) and Albumin (Alb) of the Studied Groups

Parameter	Group	Controls (Group I) (n=20)	HCV Patients (Group II) (n=20)	HCC Patients (Group III) (n=30)
FSG	Median	89	102*	98.5*
(mg/dl)	Min-Max	76-97	59-347	66-205
	P		0.018	0.03
	P$_1$			NS
Creatinine	Median	0.9	1.1*	1.15
(mg/dl)	Min-Max	0.8-1.3	0.7-3.9	0.8-5.9
	P		0.027	NS
	P$_1$			NS
Total Protein	Median	7.65	7.3	6.9*
(g/dl)	Min-Max	6.8-8.2	6.2-8.4	5.2-9
	P		NS	0.018
	P$_1$			NS
Albumin	Median	4.5	2.9*	2.85*
(g/dl)	Min-Max	4-4.9	2.3-4.7	2-3.8
	P		0.000	0.000
	P$_1$			NS

Table 3. Serum Values of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (AP), Gamma Glutamyl Transferase (GGT) Activities and Total and Direct Bilirubin of the Studied Groups

Parameter	Group	Controls(Group I)(n=20)	HCV patients (GroupII) (n=20)	HCC Patients (Group III) (n=30)
ALT	Median	16	35.5*	65*•
(U/L)	Min-Max	9-37	6-121	13-273
	P		0.003	0.000
	P$_1$			0.024
AST	Median	17	53.5*	111.5*•
(U/L)	Min-Max	11-25	10-137	21-364
	P		0.000	0.000
	P$_1$			0.003
AP	Median	81	122*	171.5*
(U/L)	Min-Max	36-97	71-408	88-401
	P		0.000	0.000
	P$_1$			NS
GGT	Median	25	48.5*	91.5*•
(U/L)	Min-Max	16-38	26-200	48-464
	P		0.000	0.000
	P$_1$			0.003
Total bilirubin	Median	0.7	1.45*	2.34*•
(mg/dl)	Min-Max	0.4-0.9	0.5-20.1	0.5-34
	P			0.000
	P$_1$			NS
Dirct bilirubin	Median	0.2	0.7*	1.3*
(mg/dl)	Min-Max	0.1-0.3	0.1-14.6	0.1-27.1
	P		0.000	0.000
	P$_1$			NS

Table 4. Serum Values of Alpha Fetoprotein (ng/ml) and Survivin (pg/ml) in the Studied Groups

Parameter	Group	Controls (GroupI) (n=20)	HCV patients(GroupII) (n=20)	HCC Patients (GroupIII) (n=30)
AFP	Median	1.85	4 *	1,222.5*•
(ng/ml) =	Min-Max	0.8-5.3	1.6-18.1	11.5-40,000
(µg/L)	P		0.000	0.000
	P$_1$			0.000
Survivin	Median	13.0	13.9*	13.9
(pg/ml)	Min-Max	11.0-16.6	12.4-62.0	11.6-125.0
	P		0.039	NS
	P$_1$			NS

had shrunken liver, 4 out of 20(20%) had hepatomegally only, 11 out of 20 (55%) had spleomegaly, 2 out of 20 (10%) had periportal fibrosis, 2 out of 20 (10%) had one focal lesion, 1 out of 20 (5%) had portal vein thrombosis and 13 out 20 (65%) had portal hypertension.

In the hepatocellular carcinoma patients group (Group III): 16 out of 30 (53.3%) had enlarged liver with cirrhotic changes, 2 out of 30 (6.6%) had shrunken liver, 4 out of 30(20%) had hepatomegally only, 16 out of 30 (53.3%) had splenomegally, 21 out of 30 (70%) had periportal

Table 5. Significant Correlations of Survivin in the Studied Groups

Group	Parameter	r_s	P
Control group	HB	-0.478	0.033
	Alb	0.484	0.031
	T. BIL	0.650	0.002
	D. Bil	0.722	0.000
HCV Group (Group II)	ALT	-0.494	0.027

fibrosis, 14 out of 30 (46.6%) had one focal lesion, 4 out of 30 (13.3%) had two focal lesions, 1 out of 30 (3.3%) had three focal lesions, 11 out of 30 (36.6%) had multiple focal lesions, 6 out of 30(20%) had portal vein thrombosis, 21 out of 30(70%) had portal hypertension and 3 out of 30(10%) had secondaries.

All control subjects were negative for *S. mansoni* . 11 out of 20 (55%)were positive for *S. mansoni* in GroupII and 22 out of 30 (73.3%) in GroupIII.

There was a significant decrease in prothrombin activity in HCVpatients (Group II) and HCC patients (Group III) when each was compared to control group (P=0.000 for both) (Table 1).

There was a significant decrease in serum level of total protein in HCC patients(GroupIII) as compared to control group (p=0.018). There was a significant decrease in serum albumin in HCVand HCC patients (Groups II andIII) when each was compared to the control group (group I) (p=0.000for both) (Table 2).

There was a significant increase in ALT, AST, AP and GGT activities in HCV patients (Group II) when each was compared to the control group and also a significant increase when HCC patients (group III) was compared to control group.But when comparing HCC to HCV patients, only ALT, AST and GGT showed significant increase.

There was a significant increase in serum level of total and direct bilirubin in HCV patients (group II) and HCC patients (group III) when each was compared to the control group (p=0.000 for all) (Table 3).

There was a significant increase in AFP in HCV and HCC patients when each was compared to the control group (p=0.000 for both). Also there was a significant increase when HCC patients (group III) was compared with HCV patients (group II) (p=0.000) (Table 4).There was a significant increase in survivin level in HCV patients (group II) when compared to the c ontrol group (group I) (p=0.039) (Table 4).

Discussion

Regulation of apoptosis (programmed cell death) is important in morphogenesis of human tissues. Impairment of apoptosis is one of the major factors contributing to carcinogenesis. Among the IAP members, Survivin is unique in that it is undetectable in the majority of normal adult tissues (Ito et al., 2000; Bokarewa et al., 2005; Ye et al., 2007). The biological functions of Survivin, other than its antiapoptotic effect, are not well understood in human cancers (Ito et al., 2000).

However, recent studies have defined a role for Survivin in regulating function in normal adult cells

suggesting that survivin disruption could have adverse consequences (Fukuda et al., 2006).

Hepatocellular carcinoma (HCC) accounting for about 4.7% of chronic liver disease in Egypt (Chiou et al., 2007). Several risk factors for HCC include cirrhosis, hepatitis B and C infections, alcohol abuse, aflatoxin exposure, hemochromatosis and alpha-1 antitrypsin deficiency (Belefer et al., 2002).

It has been reported that not only increased cellular proliferation but also diminished cell death play important roles in hepatocarcinogenesis and tumor progression in HCC (Takehara et al., 2001; Ikeguchi et al., 2002).

Nowadays, there is a need for a biochemical tumor marker that could do better, instead of relying on the traditional marker, AFP with its fallacies in the screening, diagnosis and prognosis of a great number of HCC cases.

To our knowledge, survivin expression has been studied at cellular level and in tissues in patients with HCC but little is known about its serum level in such a disease (Ye et al., 2007).

The aim of the present work is to evaluate the serum level of Survivin in patients with hepatocellular carcinoma associated with hepatitis C infection and to compare it with its level in patients with chronic hepatitis C infection. The diagnostic performance of survivin, as a possible tumor marker, is compared to that of serum AFP.

In the present study, the median value of serum Survivin in the control group was 13 pg/ml (range 11-16.6 pg/ml (Bokarewa et al., 2005) found that the plasma level of Survivin in their controls was 121±2 pg/ml (mean±S.D). Also, (Derin et al., 2008) found that Survivin level in their control group have median value equal 36.2 pg/ml, range equal to 11.6-106 pg/ml. (Gosksel et al., 2007) found that Survivin level among their control group to have median value equal to 68.9 pg/ml, range equal to53.6-102.9 pg/ml. This variability in Survivin level in healthy adults could be attributed to differences in the groups chosen as regards age, sex, and ethnicity .

The median serum value of Survivin in the hepatitis C viral infection group (group II) was 13.9 pg/ml (12.4-62 pg/ml). There was a significant increase in Survivin level in HCV patients group (group II) when compared to the control group (group I) (p=0.039). (Table 4) This could be explained by increase apoptosis of hepatocytes due to HCV viral infection.

In hepatocellular carcinoma group (group III), Survivin was13.9 pg/ml (11.6-125 pg/ml). There was no significant difference in HCC patients group (group III) when compared to control group (group I) or between HCC group (group III) and HCV group (group II). From this observation, it could be shown that apoptosis due to HCV infection, decreases by time as the disease progresses.

At tissue level, (Zhu et al., 2005) found that there was a remarkable increase in Survivin expression between the HCCtumor tissue and liver cirrhosis tissues (P<0.01). Survivin protein was detected in 23 (60.5%) of 38 HCCs and 3 (7.9%) of 38 liver cirrhosis tissues. The expression of survivin in HCC was related to the metastasis of HCC (P<0.05) by immunohistochemistry and Western blot.

(Yagihashi et al., 2005) found that, in patients with

Table 6. Cut off Value, Diagnostic Sensitivity, Specificity and Predictive Value of Positive and Negative Results and Efficiency of the Test for Survivin and Alphafetoprotein (AFP)

	Cutoff value	Sensitivity %	Specificity %	Predictive value of positive test	Predictive value of negative test	Efficiency %
Survivin	13.7pg/ml	53.3%	62.5%	51.6%	64.1%	58.57%
AFP	10.85ng/ml	100%	92.5%	90.9%	100%	95.7%

chronic viral hepatitis, elevated anti-survivin antibodies were detected in 10 of 57 sera (17.5%); and in HCC patients in 7 of 29 sera (24.1%). The levels of anti-survivin antibodies in HCC patients with HCV infection were significantly higher than those in the healthy controls and HCC patients with HBV infection. However, there were no significant differences in the levels of anti-survivin antibodies between HCV and HCC patients with HCV infection.

(Montorsi et al., 2007) quantitated survivin mRNA levels by real time RT-PCR in 25 patients. They found that Survivin mRNA was documented in all liver tissues with significantly higher levels in neoplastic specimens (p=0.01). In non-neoplastic liver tissue, survivin levels were correlated with activity score of chronic liver disease. Increased Survivin levels were correlated with high tumor grade (p=0.05) and vascular invasion (p=0.005).

There was a significant correlation between survivin expression and portal vein thrombi and intrahepatic metastatic nodes (P<0.05) (Ye et al..2007).

(Cui et al., 2008) found that of the 50 HCC specimens, 32 (64.0%) were positive for Survivin expression by immunohistochemistry. Survivin expression was not detected in normal hepatic tissues. and did not correlate to the patients' gender, age, tumor size, degree of differentiation and alpha-fetoprotein level but related to the clinical stage and lymph node metastasis of HCC (P<0.05).

Regarding the work done by immunohistochemical method by (Hui et al., 2008) they found that Survivin protein was expressed in the cytoplasm in 30 of 42 cases of HCC (71.4%) and in 4 of 34 cases of adjacent cirrhosis tissues (11.8%). Expression of Survivin protein was negative in 10 cases of normal tissues. Protein positive expression rate in HCC was significantly higher than adjacent cirrhosis tissues and normal tissues (P<0.001).

As regards serum AFP which is considered a valuable tumor marker in the diagnosis and prognosis of HCC despite pitfalls in its diagnostic performance, the present work demonstrated sharply elevated highly significant serum AFP in the HCC group compared to the other two groups (p=0.000 for both). Furthermore, despite being within the safely reported reference intervals, yet the present work demonstrated a significantly higher AFP median value in HCV group compared to the control group. This goes in concordance with many studies reporting the same finding in HCC and HCV groups compared to the control group (Takehara et al., 2001; Ezzat S et al., 2005).

(Tong et al., 2001), pointed to many pitfalls of AFP as a tumor marker, particularly in its degree of diagnostic specificity and sensitivity, being altered in benign and malignant conditions. The pursuit for a potentially new biochemical marker for the prediction and diagnosis

of HCC was carried out in the present work by the measurement of plasma Survivin.

(Filmus et al., 2004) found that the only serological marker currently widely used for the diagnosis of HCC is AFP. However, the sensitivity of this marker is limited (41-65%). Given the high heterogeneity of HCC, it is currently thought that an optimal serological test for HCC will be based on the simultaneous measurement of two or three highly specific serological markers .

When comparing the diagnostic performance of plasma Survivin as a possible tumor marker in HCC to the well established HCC marker serum AFP, receiver operating characteristics (ROC) curves were done and the best generated cut off values for both AFP and survivin were 10.85 ng/ml and 13.7pg/ml respectively. We came to a conclusion that serum AFP still surpassed plasma survivin due to the higher degree of both diagnostic specificity , sensitivity, positive and negative predictive value as well as efficiency (se Table 6).

From the present work it could be concluded that : 1) Serum Survivin showed higher values in HCV group compared to controls; 2) Serum AFP showed a better diagnostic performance and proved to be more reliable as a tumor marker for HCC.

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