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Estimation of Des-gamma-carboxy prothrombin with Alpha Fetoprotein Elevates the Diagnostic Performance of Hepatocellular Carcinoma among Upper Egyptian Hepatitis C Patients

Nabil Mohie Abdel-Hamid*, Abdel-Hamid NM, Wahid AM, Anbar NH and Helaly T

*Department of Biochemistry, College of Pharmacy, Kafrelsheikh, Minia Universities, Kafrelsheikh and Miniaand Mansura University Hospital, Mansura, Egypt.

ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth most frequent neoplasm and the third most common cause of cancer-related death in the world. This study aims to evaluate the usefulness of des-gamma-carboxyprothrombin (DCP) with alpha fetoprotein (AFP) in early speculation of HCC, through comparing the levels of DCP and AFP in three equal groups (20 individuals/ each), HCC, chronic HCV patients+/-interferon-α2+ribavirin (IF-α2 +RV) treatment, to normal healthy individuals (20 individuals). This, to evaluate if the DCP can be accredited as a better diagnostic tool for HCC patients. All cases were subjected to the following investigations: routine liver function tests, HCV antibody titer, serum levels of AFP and plasma DCP. The results revealed that sensitivity of DCP was higher than AFP in detecting HCC (85% versus 65% of AFP) whereas the specificity of DCP was lower than that of AFP (72.5% versus 97.5% for AFP) among HCV patients. Recommendation, these results suggest that regular screening for HCV is a must, being a global risk factor for HCC, as well as, simultaneous estimation of both AFP and DCP in an attempt to allow early diagnosis of HCC.

Keywords: Alpha fetoprotein, Diagnosis, Des-gamma-carboxyprothrombin, Hepatitis C virus, Hepatocellular carcinoma, Interferon-α2+Ribavirin.

Corresponding author

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent human cancers worldwide, with 1 million of newly diagnosed cases each year. Although knowledge about HCC is expanding exponentially, treatment and prevention of HCC is still a big challenge, and requires thorough understanding of the molecular mechanisms of hepatocarcinogenesis. This disease is responsible for more than 500, 000 deaths per year. The incidence ranges from <10 cases per 100, 000 persons in North America and Western Europe to 50-150 cases per 100, 000 persons in parts of Africa and Asia, where HCC is responsible for a large number of cancer deaths[1].

Approximately, 75–80% of primary liver cancers are attributable to persistent viral infections. HCC develops in more than 80% of cases in cirrhotic livers with an annual incidence of 2-5%. Cirrhosis is the major risk factor for hepatocellular carcinoma and cirrhotic patients should be screened for early detection of HCC [2, 3]. Although UltraSonography (US) and conventional tumor markers such as α-fetoprotein are widely used for HCC detection in clinical scenes, they still do not provide an entirely satisfactory solution to detect HCC at the early stage. For instance, abdominal USis the only tool recommended to screen patients with cirrhosis at present. However, its sensitivity in detecting small HCC (namely those below 1-2 cm) is rather low. The strongest information predicting prognosis and thus also possibly driving treatment strategy, are obtained, in fact, only from histological examination of the tumor on a resected specimen, which clearly is not available at the time of diagnosis [4].Surveillance programs are therefore recommended for the early detection of HCC, aiming at increasing the number of patients suitable for curative treatments. This points out the urgentneed for new surveillance tools of patients at risk, mainly cirrhotic patients, for detection of HCC in the early stage.

Serum alpha fetoprotein (AFP) is the most widely used tumor marker in diagnosing patients with HCC and has been proven to have capability of prefiguring the prognosis. Furthermore, some other tumor markers, such as glypican-3, gamma-glutamyl transferees II(GGT), alpha-L-fucosidase, transforming growth factor-beta 1 and tumor-specific growth factor, have been indicated to be supplementary to AFP in the detection process. AFP mRNA has shown to correlate with the metastasis and recurrence of HCC, and it may be the most useful marker to prefigure the prognosis, some other markers, such as gamma-glutamyltransferase mRNA, vascular endothelial growth factor, and interleukin-8, could also be used as prognostic indicators and the simultaneous determination of AFP with these markers may detect the recurrence of HCC at its earlier period[5, 6].Alpha fetoprotein (AFP) is a fetal specific glycoprotein produced primarily by the fetal liver. Normally, its serum concentration falls rapidly after birth and its synthesis in adult life is repressed. However, greater than 70% of HCC patients have a high serum concentration of AFP because of the tumor excretion. Serum AFP remains the most useful tumor marker in screening HCC patients. The serum concentration of 20 ng/ml, is the most commonly used cut-off value to differentiate HCC patients from healthy adults. However, some investigations have showed that the cut-off value is fluctuant in different ethnic groups [7].On the other hand some reports have indicated that it has limited utility of differentiating HCC from benign hepatic disorders for its high false-positive and false-

negative rates and patients with acute exacerbation of viral hepatitis but no HCC may also have markedly increased AFP levels[8].

Des-gamma-carboxyprothrombin (DCP), a protein induced by vitamin K absence or antagonist II (PIVKA-II), is an abnormal product from liver carboxylation disturbance during the formation of thrombogen. Its mean serum concentration, which is not correlated to serum levels of AFP, is obviously elevated in HCC patients compared to healthy adults and patients with nonmalignant hepatopathy. Serum and tissue DCP have been proved to be more useful than AFP in differentiating HCC from nonmalignant hepatopathy and in detecting patients with small HCC [9].The sensitivity and specificity of serum DCP (at the cut-off value of 125, ng/ml) in discriminating HCC from nonmalignant hepatopathy were 89% and 86.7% respectively, which were much better than those of AFP (at the cut-off value of 11 ng/ml). Furthermore, the simultaneous determination of DCP and other tumor markers, such as AFP and AFP-L3 may have a greater accuracy than the determination of each of them alone[10]. Besides the purpose of screening HCC, serum DCP could also be used as a prognostic indicator for HCC patients and may be more useful than AFP in reflecting the invasive characteristics of HCC. It has been reported that patients with DCP seropositive and AFP seronegative have a higher frequency of HCC with a distinct margin, large nodule more than 3 cm. Moreover, the simultaneous of serum DCP levels and tissue DCP expression is more valuable than either factor alone in pursuing the prognosis of patients [11].

The present study aims to evaluate the accuracy of DCP as a tumor marker in diagnosing HCC, compare the level of DCP and AFP in HCC patients, in chronic HCV patients +/- interferon-α2+ribavirin (IF-α2 +RV) treatment, define the level of each tumor marker with the best sensitivity and specificity for HCC diagnosis and finally, evaluate if the combination of DCP with AFP gives better diagnosis for HCC.

PATIENTS AND METHODS

Patients:

This study was conducted as a collaboration between the Department of Biochemistry, College of Pharmacy, Minia University and Sohag Cancer Center at Sohag Health Insurance Hospitals, South Egypt, between January 2009 to February 2011. The study included 80 individuals grouped as follows:

Group 1: 20 healthy volunteers, gender matched to other groups with the following clinical conditions: apparently healthy, normal clinical examination, abdominal ultrasonography, liver function tests and seronegative for HCV markers.

Group II: 20 patients with HCC regardless the etiology with the following clinical diagnosis: deterioration of health, right hypochondrial pain and hepatomegaly with nodular surface, the abdominal ultrasonography showing hepatic focal lesions (single or multiple) or heterogeneous areas in the liver. Positive histopathological examination of liver biopsy or aspirate for

malignancy (only when the patient's clinical condition and prothrombin time and concentration allowed the performance) and/or raised AFP above 400 ng/ml.

Group III:20 patients with chronic HCV, matched in gender to group I with the following clinical conditions: fatigue, anorexia, with high aminotransferase values and hyperbilirubinemia. They were not under interferon-α2+ribavirin (IF-α2 +RV) treatment.

Group IV: 20 patients with chronic HCV, under IF-α2 (weekly subcutaneous single dose, 160 ug/ampoule ,)plus RV (1200 mg/ day, *per os* doses after meals divided into 3 doses)for one year: this group of patients matches to group I in gender, with the same diagnosis as group III. Both groups III and IV were subjected to HCV RNA quantitation, all exceeded a million copy/ml serum, those who suffered side effects or lack of response to IF--α2 + RV were assigned as group III, who holders of promising response to therapy were assigned as group IV.

All cases were subjected to the following investigations: complete history of patients attending Sohag Cancer Center at Sohag Health Insurance Hospitals through patient files, full clinical examination, including general and abdominal examination with stress on the size of the liver and spleen. Abdominal US was performed to assess the status of the liver and spleen to detect the presence of ascites. Ultrasonographically guided percutaneous needle biopsy from the liver was taken. Laboratory investigations included liver function tests with the aim of investigating the status of the liver in all patients and to exclude liver disease in control subjects. Liver function tests included serum bilirubin both total, direct and indirect, serum albumin, AST, ALT activities, Prothrombin time (PT) and concentration. Hepatitis C viral antibodies were detected by automated ELISA. Serological tests for HCV markers were performed to assess the possible role of the virus in the etiology of HCC. Serum AFP, Plasma DCP were finally measured.

Sample Collection:

All samples for HCV infected subjects were taken after one year of recording the infection, whether toke the combination therapy or not. Tenmls venous blood were withdrawn from each individual, 1.8 ml were immediately mixed with 0.2 ml tri-sodium citrate for estimation of PT and concentration. The rest of the blood was divided into 2 portions, one smaller, anti-coagulated for DCP estimation, the rest left to clot and centrifuged afterwards for other investigations. The rest of plasma and serum were separated in aliquots and frozen at -70ºC for measurement of DCP and AFP.

HCV Antibody Assay:

Micro plates coated with HCV-specific synthetic antigens derived from "core" and "NS" regions encoding for conservative immunodominant antigenic determinants (core, NS3, NS4, and NS5) were used for this assay according to manufacturers' instructions(Ortho HCV Core-Ab Irma Test; Mitsubishi Kagaku Iatron, Japan).

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AFP (A1-Fetoprotein):

Sandwich principle was employed to determine the AFP concentration via ELISA technique according to manufacturers' instructions (Anogen, Canada) [12-14].

Des-Gamma-Carboxyprothrombin:

DCP was measured using commercially available ELISA kit (Asserachrom PIVKA II kit, Stago, France), according to the manufactures' instructions[15, 16].

The Cut-Off value was calculated using the following formula[17]:

Negative Control + 0.200 =cut-off, samples with an optical density (OD), (450 nm) value below the cut-off were considered negative for anti-HCV antibodies.

Samples with OD (450nm) within the range of cut-off + 20% are considered to be in the Grey Zone. Patients require follow up, however, results just below the cut-off values (Vs 10%) were be interpreted with care. Sample with an OD greater than or equal to the cut-off value were considered to be initially positive &and retested in duplicate before final interpretation.

After retesting, the sample was considered to be positive if at least one of the two values is positive, i.e. higher than, or equal to the cut-off value. The sample was considered to be negative if both values are less than the cut-off value. Samples which have been retested twice and found negative but with one value near the cut-off value (Vs 10%) were interpreted with care. Samples with an OD (450 nm) value above the upper limit of the Grey Zone were considered positive for anti-HCV antibodies.

Calculation of sensitivity and specificity of AFP and DCP:

Sensitivity: the capacity of the test to correctly identify affected individuals in a population "TRUE POSITIVES". The greater the sensitivity, the smaller the number of identified case "false negatives".

Sensitivity= TP/ (TP+FN)

where , TP = true positive, and FN = false negative

Specificity:

The capacity of the test to correctly exclude individuals, who are free of the disease "TRUE NEGATIVES". The greater the specificity, the fewer "false positives" will be included.

Specificity= TN/ (TN+FP)

Where, TN = true negative, and FP = false positive.

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Statistical Analysis:

The results were presented as group means ± SD and statistically significant differences between mean values were determined by one way analysis of variance (ANOVA). Values of $P<0.05$ were considered statistically significant. Graph Pad Prism v.5.0 software was used for this analysis.

RESULTS

In the present study, the male/ female percentages and age ranges are shown in Table 1.

Table 1: Age and sex variations in the selected groups of patients and the control (Mean ± S.E, N=20)

		Group I(Control)	Group II (HCC)	Group III (Non treated HCV patients)	Group IV(Treated HCV patients)
Age (years)		49±3	55±2	45.2±1.4	46.5±1.3
Sex	**Female%**	20	35	10	10
	Male%	80	65	90	90

Liver function tests in patients infected with chronic HCV and HCC:

ALT and AST serum activities were significantly ($P< 0.001$) up-regulated in the HCC and HCV groups (groups II, III, IV), compared to control (group I), (Table 2).

Table 2: Liver function tests of all groups of patients and the control (Mean ± S.E, N=20):

	Group I (Control)	**Group II (HCC)**	**Group III (Non treated HCV patients)**	**Group IV (Treated HCV patients)**
ALT (U/L)	27.0±2.1	70.2±14.0**	92.3±17.0***	70.0±13.3**
AST (U/L)	22.0±1.0	99.0±15.6***	81.2±11.4***	59.8±9.4**
Albumin (g/dl)	4.0±0.1	1.40±0.9***	4.0±0.2	3.4±0.10**
Total bilirubin (mg/dl)	0.5±0.04	1.9±0.24***	1.2±0.3**	1.04±0.08*
Direct bilirubin(mg/dl)	0.15±0.01	0.95±0.15***	0.50±0.1**	0.36±0.03*
PT(Seconds)	11.6±0.05	14.81±0.6***	14.40±0.3***	14.3±0.33***
PC	92.3±0.76	71.8±3.9***	69.6±2.1***	83.2±3.3*

All groups are compared to control. NS: $P>0.05$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

A highly significant difference in total and direct bilirubin in groups II, and III respectively, compared to control. Moreover, HCC patients (group II) showed a non significant decrease in serum albumin, compared to control. The mean prothrombin time (PT) in HCC group and non-malignant HCV groups (III, IV) showed a highly significant elevation, compared to control. Regarding the prothrombin concentration(PC), a highly significant decrease in group (II, III, IV), was shown. Moreover, 85.6% of the patients in the HCC group included in this study were reported to have HCV infection (Table 4).

Table 4: DCP and AFP sensitivity and specificity in HCC versus nonmalignant groups (III and IV):

Cut-off (ng/ml)	Sensitivity %	Specificity %
DCP <12	85	72.5
AFP <121	65	97.5

Alpha fetoprotein (AFP) and Des-gamma-carboxyprothrombin (DCP):

Both AFP and DCP serum values of HCC patients showed highly significant increase, compared to non-malignant groups (I, III, IV) (Table 3). Our results also showed that the sensitivity of DCP was higher than AFP in detecting HCC patients (85% versus 65% of AFP). In our data, the increase in sensitivity of DCP from 85% and AFP from 65% to 90% (Table 4) in detecting HCC was found by the complementary use of the two markers.

DISCUSSION

The increasing incidence of HCC, compounded by the fact that the majority of these tumors were diagnosed at a late stage when curative treatment were not possible[18], has prompted the international community into performing regular surveillance of high risk individuals. Unfortunately, surveillance programmers are hindered by the poor performance of the commonly used serum marker, namely AFP [19]. Even in combination with abdominal US. A tremendous effort has been applied to the search for improved HCC biomarkers. As yet, none has proved superior to AFP in performance, but in combination some may have complimentary roles in HCC arising on a background of viral hepatitis [20].

Sex and age of patients included in this study:

In the present study, the male to female among the HCC group (Table 1), was comparable to other reports elsewhere[20, 21].In Europe, the ration ranges from 4 to 5: 1, while in, 3.2:1, Japan,3.7:1, and in Gambia 2.8:1. Men are more likely to consume alcohol, smoke cigarettes, and have increased iron stores. Non-environmental endogenous factors that may also affect males adversely such as higher body mass index and higher levels of androgenic hormones[22, 23].

HCC affects all ages. In Asian and African countries, the morbidity peak is reached in adolescence (20 and 40 years). HCC has also been observed in babies and infants [24, 25].The mean age of HCC is significantly higher than those of chronic hepatitis patients with and without interferon treatment, which is similar to some other reports [26].

Liver indices in tested groups:

ALT and AST activities were increasedin HCC and HCV groups, compared to the control group. These results were comparable to previous reports [27].Both serum total and direct bilirubin, theywere significantlyelevated in HCC and non-treated HCV, compared to control group.Moreover, HCC patients (group II) showed a significant decrease of serum

albuminrelative to the control group, reflecting a decreased synthetic ability of the liver cells. These results coincide to some previous observations[28].

HCC group (II)and nonmalignant hepatitis C groups (III and IV) showed a highly statistically significant elevation in the mean PT with corresponding PC depression, comparing with control group (I). These results are comparable to the findings of Raedle etal.,[29].

HCV antibody and AFP testing:

In the present study 85.6% of the patients in the HCC group were referred to have HCV infection. Similar results were found elsewhere; as 70% [30, 31], 84% [32], 75.8% [33, 34] and 65%[35] in Japan. Lower rates were found in some other areas in Egypt [36], where HCV prevalence among HCC patients was found to be 30%.This discrepancy in prevalence rates might be due to cultural and hygienic difference in individuals contributing in the surveillances.

There was an increased risk of HCC accompanying HCV infection[37]. In the present study, most of HCC patientswere seropositive to HCV. This could be explained on the basis that chronic hepatitis always progresses to cirrhosis, ultimately developing HCC[38].It was shown, that in spite of the pitfalls of using AFP as a marker of HCC, it is commonly used as one of the screening tools in combination with ultrasonography[39]. Though some clinicians find AFP to be of no use for screening. Thus, Tremolola et al.[40]demonstrated that the sensitivity can reach up to 100% by combining AFP measurement and ultrasonography.

In our work, AFP values in control group were 150% higher than conventional values in other regions of Egypt, also, other tested groups. HCC patients(II) showed highly significant increase, rather than the nonmalignant groups (Table 3).

Table 3: Alpha fetoprotein and des-gamma carboxyprothrombin blood levels in groups of patients against control group (Mean ± S.E, N=20)

	Group I (Control)	Group II (HCC)	Group III (Non treated HCV patients)	Group IV(Treated HCV patients)
Serum AFP (ng/ml)	32.25±3.6	924.7±92.2***	629.9±69.5***	321.8±27.4***
Plasma DCP (ng/ml)	1.21±0.08	216.3±49.6***	175.3±32.6***	118.8±26.5***

All groups are compared to control. *** P<0.001.

Yuen and lay [39]showed that AFP levels do not correlate with tumor size, histological grades, intrahepatic metastases and portal vein thrombosis. The elevated AFP values among chronic HCV patients still a promoter for looking for alternative markers do discriminate HCC from chronic HCV interference. This implication was recently documented by Richardson et al., who reported that AFP and ALT values correlatein patients with chronic HCV infection; however, among patients with HCC, levels of AFP increase disproportionately to/or

unaccompanied by increases in levels of ALT. The prognostic and diagnostic value of AFP levels might be increased by adjusting ALT values[41].

Des-gamma-carboxyprothrombin (DCP), a protein produced in the absence of vitamin K, an abnormal product from disturbed carboxylation during the formation of prothrombin. In fact, many reports stated that it is more specific for HCC than AFP and is less often elevated in cirrhotic patients without HCC. Thus, the combination of DCP with AFP might be even more sensitive and specific[10, 26].

Yuen and Laiconcluded that DCP is an excellent marker for monitoring the treatment efficacy, indication of complete clearance of HCC after curative treatment, and recurrence of HCC in the future. The serum half-life of DCP is around 40-70 hours, much shorter than that of AFP,5-7 days. Unlike AFP, DCP is found to correlate with the stage of the HCC as well as survival[39].

In the present study, DCP values of HCC group showed a highly significant increase upon comparison with the non malignant groups (I, III, and IV) (Table 3). The sensitivity of DCP was higher than AFP in detecting HCC patients (85% versus 65%), a result quite similar to previous report[26]. Some studies reported that the unsatisfactory performance of AFP in the diagnosis of HCC, was due to the high false positive and false negative results [19].On the other hand, our results showed specificity of DCP lower than that of AFP (72.5% versus 97.5%), comparable to elsewhere statement[42].

In our data, the increase in sensitivity of DCP and AFP (Table 4) in detecting HCC was found by the simultaneous use of the two markers. Similar results was reported[43], suggesting that DCP and AFP together could be useful for the evaluation of tumor progression, prediction of patient outcome and treatment efficacy. Fujiyama[44]concluded that since DCP in serum has half life of about 40-70 hours, it can reflect the therapeutic efficacy on HCC much more promptly than AFP which has a half life of 5-7 days, and the mutual use of both may raise diagnostic performance. We found little correlation between DCP and AFP in HCC patients. This finding was similar to previous studies[45]which showed that no correlation exists between DCP and AFP and the combined measurement of these two markers appears to be useful in the diagnosis of hepatocellular carcinoma. Yoshida[46] concluded that AFP and DCP may reflect the different biological characteristics of HCC and the combination of both markers may be necessary for evaluating the clinical states of HCC.

Conclusively, the simultaneous determination of both markers improved the sensitivity of the diagnosis of HCC in HCV patients. Although the specificity of AFP seems higher than that of DCP, the higher sensitivity of the latter potentiates the need for co determination of both markers to reach more efficient diagnosis of HCC among HCV carriers as sensitivity issue is greatly looked for in risky cases.

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REFERRENCES

[1] Parkin DM. Int J Cancer 2006; 118: 3030-3044.
[2] Rabe C, Cheng B, Caselmann WH. Dig Dis 2001; 19: 279-287.
[3] Shi J, Zhu L, Liu S, Xie WF. Br J Cancer 2005; 92: 607-612.
[4] Jain S, Singhal S, Lee P, Xu R. Am J Transl Res 2: 105-118.
[5] Abdel-Hamid N. IJIB 2008; 3: 196-2001.
[6] Zhou L, Liu J, Luo F. World J Gastroenterol 2006; 12: 1175-1181.
[7] Soresi M, Magliarisi C, Campagna P, Leto G et al. Anticancer Res 2003; 23: 1747-1753.
[8] Nguyen MH, Garcia RT, Simpson PW, Wright TL, Keeffe EB. Hepatology 2002; 36: 410-417.
[9] Gotoh M, Nakatani T, Masuda T, Mizuguchi Y et al. Jpn J Clin Oncol 2003; 3: 522-526.
[10] Okuda H, Nakanishi T, Takatsu K, Saito A et al. Cancer 2000; 88: 544-549.
[11] Motola-Kuba D, Zamora-Valdes D, Uribe M, Mendez-Sanchez N. Ann Hepatol 2006; 5: 16-24.
[12] Chan DW, Miao YC. Clin Chem 1986; 32: 2143-2146.
[13] Abelev. G I 1974; 20: 3-37.
[14] Hirai H, Nishi S, Watabe H. 1973; 14: 19-34.
[15] Amiral J GM, Plassart V, Mimilla F, Corbel L, Chambretie B, Blood Coagul Fibrinolysis 1990; 1: 739-740.
[16] Hirschauer C, Amiral J, Marcellin P, Guillin MC. Bezeaud A 1991; 65: 1056.
[17] Ridge SE, Vizard AL. J Clin Microbiol 1993; 31: 1256-1261.
[18] Wilson JF. Ann Intern Med 2005; 142: 1029-1032.
[19] Bruix J, Sherman M, Llovet JM, Beaugrand M et al. J Hepatol 2001; 35: 421-430.
[20] Giannelli G, Fransvea E, Trerotoli P, Beaugrand M et al. Clin Chim Acta 2007; 383: 147-152.
[21] Gomaa AI, Khan SA, Toledano MB, Waked I, Taylor-Robinson SD. World J Gastroenterol 2008; 14: 4300-4308.
[22] El-Serag HB, Rudolph KL. Gastroenterology 2007; 132: 2557-2576.
[23] Badawi AF, Michael MS. Anticancer Res 1999; 19: 4565-4569.
[24] Yamazaki Y, Kakizaki S, Sohara N, Sato K et al. Dig Dis Sci 2007; 52: 1103-1107.
[25] McGlynn KA, Tsao L, Hsing AW, Devesa SS, Fraumeni JF. Jr Int J Cancer 2001; 94: 290-296.
[26] Wang CS, Lin CL, Lee HC, Chen KY et al. World J Gastroenterol 2005; 11: 6115-6119.
[27] Lopez JB, Balasegaram M, Thambyrajah V, Timor J. Malays J Pathol 1996; 18: 95-99.
[28] Rabe C, Pilz T, Klostermann C, Berna M et al. World J Gastroenterol 2001; 7: 208-215.
[29] Raedle J, Oremek G, Truschnowitsch M, Lorenz M et al. Eur J Cancer 1998; 34: 1198-1203.
[30] Colombo M, de Franchis R, Del Ninno E, Sangiovanni A et al. N Engl J Med 1991; 325: 675-680.
[31] Nishioka K, Watanabe J, Furuta S, Tanaka E et al. Cancer 1991; 67: 429-433.
[32] Mabrouk, G. M., Dis Markers 1997, 13, 177-182.
[33] Hassan MM, Zaghloul AS, El-Serag HB, Soliman O, et al. J Clin Gastroenterol 2001; 33: 123-126.

[34] Montalto G, Cervello M, Giannitrapani L, Dantona F, et al. Ann N Y Acad Sci 2002; 963: 13-20.
[35] Lopez JB. Clin Biochem Rev 2005; 26: 65-79.
[36] Darwish MA, Faris R, Darwish N, Shouman A, et al. Am J Trop Med Hyg 2001; 64: 147-153.
[37] Anthony PP. Histopathology 2001; 39: 109-118.
[38] Rahman El-Zayadi A, Abaza H, Shawky S, Mohamed MK, et al. Hepatol Res 2001; 19: 170-179.
[39] Yuen MF, Lai CL. Best Pract Res Clin Gastroenterol 2005; 19: 91-99.
[40] Tremolda F, Benevegnu L, Drago C, Casarin C, et al. Hepatogastroenterology 1989; 36: 519-521.
[41] Richardson P, Duan Z, Kramer J, Davila JA, et al. Clin Gastroenterol Hepatol 2012; 10: 428-433.
[42] Durazo FA, Blatt LM, Corey WG, Lin JH, et al. J Gastroenterol Hepatol 2008; 23: 1541-1548.
[43] Toyoda H, Kumada T, Kiriyama S, Sone Y, et al. Clin Gastroenterol Hepatol 2006; 4: 111-117.
[44] Fujiyama S, Tanaka M, Maeda S, Ashihara H, et al. Oncology 2002; 62(1): 57-63.
[45] Cui R, Wang B, Ding H, Shen H, et al. Chin Med J (Engl) 2002; 115: 42-45.
[46] Yoshida S, Kurokohchi K, Arima K, Masaki T, et al. Int J Oncol 2002; 20: 305-309.