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2'-Deoxycytidine As A Potential Biomarker For Detection Of Heptocellular Carcinoma

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Abstract:

Background: 2'-deoxycytidine (Dcyd) is one of four major nucleosides found in the different normal body fluids due to dissolution of dead cells, and is increase in the presence of malignancy. Previous studies proved that it can be used as a marker for bladder cancer and acute lymphoblastic leukemia. The aim of this study is to assess 2'Dcyd as a possible biological marker in hepatocellular carcinoma (HCC).

Methods: Four groups were evaluated for the level 2'-Dcyd as well as alpha-fetoprotein (AFP); a control group (n = 20), 20 cases of chronic liver diseases (CLD), 20 cases of hepatitis C (HCV) 60 cases of HCC.

Results: In the patients with HCC, 2'-Dcyd serum level was 8-fold higher than normal level. It was 3-fold higher in HCV group. A mild increase was noted in patients with chronic liver diseases. Levels ≥ 0.14 of 2'-Dcyd had a sensitivity of 93% and specificity of 90% for diagnosis of HCC. It also recorded a sensitivity and specificity of 90% for diagnosis of HCV.

Conclusions: For diagnosis of HCC, 2'-Dcyd is no better than AFP, as it is elevated in viral hepatitis C. A combination of AFP and 2'-Dcyd could provide broader information in diagnosis and treatment decision.

Introduction:

Worldwide, hepatocellular carcinoma (HCC) is one of the most common malignancies associated with poor prognosis (Okano *et al*., 2001). According to recent reports, the incidence of HCC has increased sharply in the last 5-10 years (El-Serag, 2002; Velazquez *et al*., 2003; Bruix *et al*., 2001). In USA, the rate of HCC has increased by 70% over the last two decades. Registry data in Canada and Western Europe show similar trends (Yu *et al*., 2000). In Egypt, El-Zayadi *et al*. (2005) reported a rising trend of HCC with increasing risk among HCV-infected men of older age groups. They recommended; careful followed-up of patients and screening for early detection of HCC. In Egypt, HCC was reported to account for about 4.7% of chronic liver disease (CLD) patients (El-Zayadi *et al*., 2001).

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world (El-Serag, 2002) complicating liver cirrhosis in most cases. Its incidence is increasing worldwide ranging between 3% and 9% annually (Velazquez et al., 2003). The epidemiology of HCC is characterized by marked demographic and geographic variations. It is the third leading cause of cancer mortality in Sub-Saharan Africa and in the Far East and ranks second in China (Shiratori *et al*., 2001; Bosch *et al*., 1999; Schafer & Sorrell, 1999).

More than 80% of HCC occurring worldwide is felt to be associated with chronic viral hepatitis (Donald *et al*., 2000).

Hepatitis virus is the major cause of acute and chronic hepatitis, leading to progressive development of necroinflammatory changes in the liver, which can result in cirrhosis and hepatocellular carcinoma (Bryant et al., 2001; Hoofnagle *et al.,* 1997) Egypt is a developing country where hepatitis B and C infection are still prevalent, the risk factor for viral transmission that specifically sets Egypt a part of other countries is a personal history of schistosomal infection (Huynh *et al.,* 2002). Specific factors important for pathogenesis of HCC are incompletely understood, and treatment outcomes have generally remained poor (Actor *et al*., 1993).

Routine surveillance of persons at risk can result in successful treatment of HCC that is detected early with screening techniques. Both periodic serial determination of alpha-fetoprotein (AFP) and hepatic ultrasound (US) examination, alone or separately, have been used successfully resulting in a higher chance of successful treatment. Alpha-fetoprotein (AFP) is one of the earliest recognized oncofetal markers. It is produced in large amount by the fetal liver, but its expression reduces sharply at birth. AFP is synthesized by most hepatoblastomas and approximately half of hepatocellular carcinomas (Taketa *et al*., 1990), and widely used in differential diagnosis and follow-up of patients with liver tumors, but so far no correlation has been found between the clinical behavior and AFP production in HCC (Lee *et al*., 1991; Yao *et al*., 1999).

Tarao *et al*. (1999) studied the correlation between DNA synthesis of heptocytes and the development of HCC in vitro, they reported that HCC tended to develop or detectable when DNA synthesis of heptocyte was increased. 2'-deoxycytidine (Dcyd) is a major modified neuclosides found in the body fluids, the source of Dcyd is not only tumor cells but also the host liver cells can produce it via stimulating the biosynthesis of Dcyd nucleotides rather than enhanced degradation of DNA (Sozen *et al*., 1999; Staub *et al*., 1996).

Yoshioka *et al*. (1997) developed sensitive and accurate procedure for quantitative determination of Dcyd in body fluids. This method is simple and reproducible for possible use Dcyd in routine examination as a biomarker for tumor detection (Yoshioka *et al*., 1997; Michino *et al*., 1997; Omura *et al*., 1997; Aziz & Wu, 2002). Previous studies on Dcyd in the National Cancer institute (NCI) in Egypt reported that it may be used in detection of breast cancer, acute lymphoblastic leukemia and bladder cancer where it showed high sensitivity in these tumors. *In this study*, we evaluate use of serum Dcyd as a biomarker for early detection of heptocellular carcinoma to avoid invasive biopsy.

Subjects & Methods

Subjects:

This study included 120 individuals; classified into four groups:

Group 1: Twenty apparently healthy individuals to serve as reference group (12 males and 8 females) their age ranged from 24 to 40. There were volunteers from the staff members of cancer biology department, National cancer Institute, Cairo University.

Group 2: Twenty patients with chronic liver disease (14 males and 6 females) their age ranged from 34 to 63.

Group 3: Twenty patients with hepatitis C (16 males and 4 females) their age ranged from 24 to 60.

Group 4: Sixty patients with hepatocellular carcinoma (49 males and 11 females) their age ranged from 29 to 70.

Patients with hepatitis C and chronic liver diseases were selected from Al- Zahra University Hospital, Al-Azhar University in June 2004. Patients with hepatocellular carcinoma were selected from inpatients of National Cancer Institute, Cairo University during the period from March 2004 to October 2004. Patients of all groups were subjected to full clinical evaluation. Sera of these patients were investigated for liver functions tests, alpha-fetoprotein (AFP), C-reactive protein (CRP) and 2'-deoxycytidine (2'-Dcyd). Individual patient profiles were collected from medical records.

Serum 2'-Dcyd was determined according to Advanced Life Science Institute, Inc., Tokyo.

Reagents:

Anti-Dcyd antibody coated micro plate (lot 0002) 96 wells store at 4°C

Reaction Buffer (lot 0002) 200 ml store at 4°C

POD Conjugate (x 100 conc.) (lot 0002) 1 ml store at 4°C

Standard Dcyd (50 mM) (lot 0002) 0.6 ml store at 4°C

Substrate Buffer (lot 0002) 80 ml store at 4°C

OPD tablet (lot 0002) 12 tab store at 4° C

Stop solution (2N H_2SO_4) (lot 0002) 80 ml store at 4° C

Preparation of various reagents:

Reaction Buffer: pH 7.3 (1% bovine serum albumin- 10 mM EDTA- 0.1M potassium phosphate - 0.15 M NaCl-0.2% Triton X-100).

Substrate Buffer: pH 5 (0.05M phosphate Citrate Buffer).

Wash Buffer: pH 7.3 (10 mM sodium phosphate containing 0.15M NaCl and 0.05% Tween-20).

Conjugate Solution: Mix OPD conjugate 1/100 in reaction buffer.

Standard Solution: The concentration of standard solution 50 mM diluted to 3 mM with reaction buffer, then, dilute 3 mM solution serially 3 times with reaction buffer. Each standard is 0, 12.5, 33,111, 333, 1000, 3000 µM .

Stop Solution: 2N. H_2SO_4.

Other Reagents:

Anti-Dcyd monoclonal antibody.

Anti-Mouse IgG conjugated peroxidase.

O-phenylenediamine (OPD) tablets (Sigma).

Procedure:

The kit components must be brought at room temperature before beginning assay.

Plate was removed from the pouch.

The first one well of the plate used as blank. 20 µl of each standard or specimen were added into the other wells followed by 100 µl of anti-Dcyd MoAb solution.

The reaction mixture was covered by seal and incubated at 37c for 90 minutes.

The content of each well was aspirated and washed by washing solution (5 cycles of 350µl/ well including blank well).

100 µl substrate solution were added into each well including blank well.

The mixture were incubated at room temperature (20-30c) for 30-33 minutes in the dark.

100 µl stop solution were added into each well including blank well, and shake plate calmly.

Readings were performed at 492 nm using a 600-700 nm reference wavelength for less than one hour after stop reaction. The optical density of blank well is 0.000.

Prepare the calibration curve for Dcyd using the optical density of each standard Vs its log concentration. Calculate amount of Dcyd in specimens by the calibration curve.

CRP (C-reactive protein) was measured by Latex Agglutination Slide Test for CRP in serum, product No. 905041-E. Serum AFP was performed according to the package insert of the kit (Matritech, USA. AFP kit. Enzyme immune-assay).

Statistical analysis:

Statistical package for social sciences (SPSS, version 12) was used for data management. Numerical variables were presented as mean±standard deviation (SD), median, range. Qualitative variables were presented as frequency and percentage. Mann-Whitney U test was used to test difference of numerical variables between two groups while ANOVA test was used on the rank of the numerical variables to test difference between more than 2 groups, followed by Sheffe test for pairwise comparison. Chi–Square test was performed to compare qualitative variables. P value less than 0.05 was considered significant and less than 0.001 was considered highly significant. For determination of cut-off levels ROC curve was applied.

Results

Clinical characteristics of the different studied groups are presented in table 1. It was noticed that all control cases were not cirrhotic, while one third of HCC group show ++ cirrhosis.

Liver enzymes (AST, ALT) and AFP were significantly higher in the three

diseased groups compared to control group. It was noticed that levels of these markers were the highest in hepatocellular carcinoma group (table 2).

Serum levels of 2'-deoxycytidine were significantly higher in both HCV and HCC groups compared to the control group. On the contrary, in the CLD group no such a difference was observed (p = 0.112) (table 1). C-reactive protein (CRP) was positive in 81.7% of HCC group and 60% in HCV group. It was positive in 15% of cases with chronic liver disease. In the control group, all cases were CRP -ve (table 1). This difference was statistically significant (p < 0.001).

Within the HCC group, there was no difference between CRP positive and negative cases concerning the levels of liver enzymes, AFP as well as 2'-Dcyd (table 3). There was a significant correlation between detection of cirrhosis and elevation of AFP and liver enzymes. On the contrary, levels of 2'-Dcyd were not significantly different in cirrhotic cases in comparison to non-cirrhotic cases within the HCC group (table 4). There was a significant correlation between 2'-Dcyd and AFP, ALT and AST (r = 0.6, 0.5 and 0.6, respectively).

Table (1): Clinical data of the studied groups

Groups	Control (n = 20)	HCC (n = 60)	CLD (n = 20)	HCV (n = 20)	P value
Age (years)					
Mean+SD	28.3±4.6[a]	56.2±8.8[b]	62.0±9.1[b]	42±11.9[c]	
Range	21-40	36-72	43-80	24-62	< 0.001
Sex	11/9	44/16	15/5	16/4	
Males	11 (55%)	44 (73.3%)	15 (75%)	16 (80%)	0.307
Females	9 (45%)	16 (26.7%)	5 (25%)	4 (20%)	
Cirrhosis Degree					
-ve	20 (100%)	25 (41.7%)	10 (50%)	16 (80%)	
+ve	0	15 (25.0%)	10 (50%)	4 (20%)	< 0.001
++ve	0	20 (33.3%)	0	0	

Table (2): Laboratory data of the studied groups

Groups	Control (n = 20)	HCC (n = 60)	CLD (n = 20)	HCV (n = 20)	P value
ALT					
Mean+SD	8.1±2.5[a]	114.1±91.2[b]	44.6±21[c]	49.8±16.9[c]	< 0.001
Median (Range)	8 (4-12)	78 (13-394)	42 (15-92)	43 (27-80)	
AST					
Mean+SD	8.8±2.5[a]	129.1±95.6[b]	56.2±27[c]	34.7±11.7[c]	< 0.001
Median (Range)	9 (4-13)	111 (22-440)	61 (26-90)	32 (18-66)	
AFP					
Mean+SD	1.6±0.78[a]	2717.1±633.7[b]	8.5±9.2[c]	4.1±2.0[c]	< 0.001
Median (Range)	1.45 (0.6-3.2)	296.5 (2-31000)	5.0 (1.3-39)	3.25 (1.8-10)	
2'-Dcyd					
Mean+SD	0.07±0.06[a]	0.58±0.85[b]	0.14±0.06[a]	0.23±0.07[c]	< 0.001
Median (Range)	0.05 (0.004-0.21)	0.29 (0.04-5.2)	0.16 (0.03-0.23)	0.25 (0.1-0.38)	
CRP					
+ve [No. (%)]	0 (0%)	49 (81.7%)	4 (20%)	12 (60%)	< 0.001

HCC: Hepatocellular carcinoma, CLD: Chronic liver disease, HCV: Hepatitis C-Virus
2'-Dcyd: 2/-Deoxycitidine
Groups with different letters are significantly different from each other

Table (3): Relation between CRP positivity and other investigated parameters in HCC group

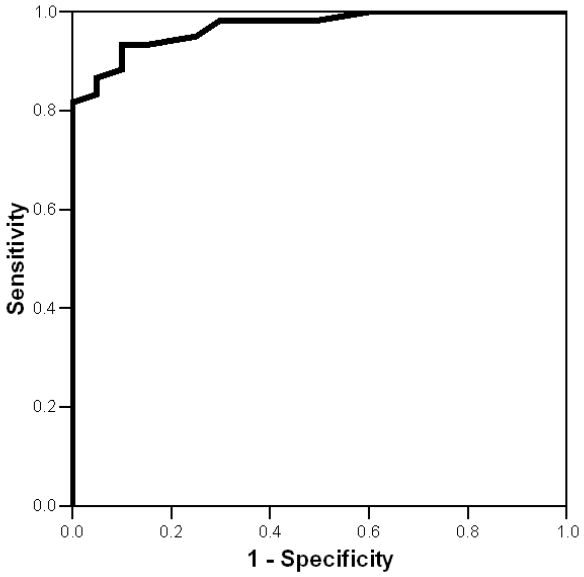
		CRP		p-value
		Negative (n = 11)	Positive (n = 49)	
2'-Dcyd	Mean±SD	0.38+0.15	0.62+0.94	0.633
	Median (Range)	0.29 (0.19-0.61)	0.29 (0.04-5.2)	
AFP	Mean±SD	3131+6905	2626+6272	0.417
	Median (Range)	1127 (4-23670)	220 (2-31000)	
AST	Mean±SD	149.9+122	125+89.6	0.709
	Median (Range)	109 (30-440)	113 (22-328)	
ALT	Mean±SD	131+105.6	110+88.3	0.379
	Median (Range)	79 (31-394)	77 (13-337)	

Table (4): Relation between cirrhosis and other investigated parameters in HCC group

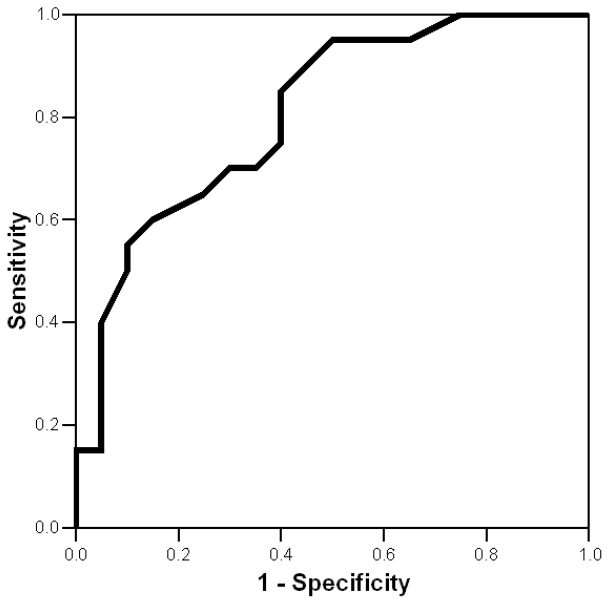
		Cirrhosis		p-value
		Negative (n = 25)	Positive (n = 35)	
2'-Dcyd	Mean±SD	0.75±1.1	0.45±0.56	0.418
	Median (Range)	0.29 (0.11-5.2)	0.29 (0.04-3.2)	
AFP	Mean±SD	1511.3±480.9	3578±717.4	< 0.001
	Median (Range)	1174 (4-31000)	21 (2-23670)	
AST	Mean±SD	85.7±97.3	160±82.4	< 0.001
	Median (Range)	52 (22-440)	125 (40-317)	
ALT	Mean±SD	87.6±101.6	133±79	0.001
	Median (Range)	48 (19-394)	112 (13-337)	

Table (5): Sensitivity and specificity for different cut-off levels of 2'-Dcyd for different diagnoses

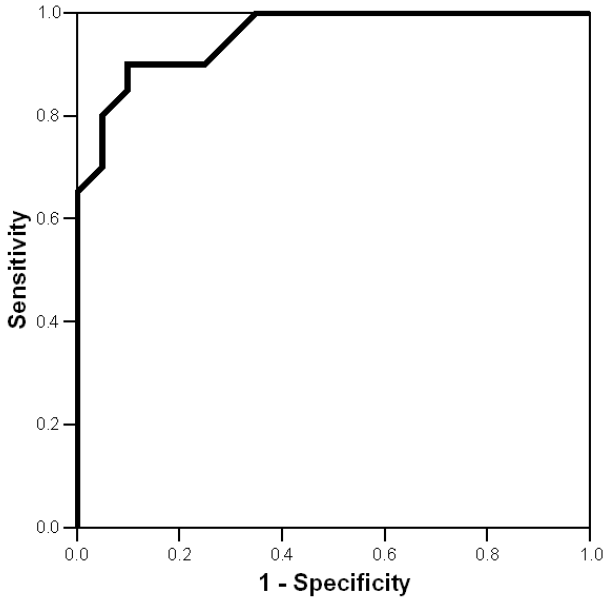
Diagnosis	Suggested Cut-Off Values	Sensitivity	Specificity
HCC	0.13	93%	85%
	0.14*	93%	90%
	0.16	92%	90%
	0.17	88%	90%
	0.18	87%	95%
HCV	0.13	90%	85%
	0.14*	90%	90%
	0.16	85%	90%
	0.18	80%	95%
CLD	0.13	60%	85%
	0.14*	55%	90%



ROC Curve for diagnosis of HCC



ROC Curve for diagnosis of CLD



ROC Curve for diagnosis of HCV

Discussion:

It is difficult to accurately diagnose early HCC. But early detection is still the only way to improve mortality. The disease is nearly fatal. The tumors are highly heterogeneous. In early-stage disease, where tumors are less than 2 cm in diameter, even expert radiologists have difficulty differentiating between a cirrhotic nodule and a malignant tumor. Liver cirrhosis is a precancerous condition that can develop into HCC (Wilson, 2005). Therefore, cirrhotic patients are usually screened for HCC during their follow-up procedure (Burtis *et al*., 2001).

Tumor markers are potential screening tools that are widely used for early diagnosis of tumors (Shu et al., 2002). Many research groups are evaluating the sensitivity of available tumor markers and also are investigating the development of a novel marker (Lim *et al.*, 2002; Yoshida *et al*., 2002; Nakatsura *et al.*, 2003). The primary marker for HCC is alpha-fetoprotein (AFP), a single polypeptide chain glycoprotein, its sensitivity and specificity are insufficient to detect HCC in all patient samples. AFP is not secreted in all cases of HCC and may be normal in as many as 40% of patients with early HCC (Di Bisceglie *et al*., 2005).

We evaluated a new biochemical factor 2'-Dcyd in addition to AFP to improve the detection of HCC and precancerous lesions hepatitis C and chronic liver diseases. 2'-deoxycytidine is one of four major nucleosides found in the different normal body fluids due to dissolution of dead cells, and it increases in the presence of malignancy and during chemotherapy (Wakui *et al.*, 1986).

The results showed elevation in liver enzymes (AST, ALT) among patients with chronic liver diseases and hepatitis C in the absence of HCC. Whereas serum AFP levels were lower in CLD and HCV patients when compared to HCC group but still significantly higher than control cases. Di Bisceglie *et al*. (2005) reported that in patients with advanced chronic hepatitis C, serum AFP values are frequently elevated, even in the absence of HCC. They also reported significant increase in serum AST and ALT among patients with chronic hepatitis C and advanced fibrosis. Similar

results were obtained by Wakui *et al*. (1986), who reported a statistically significant elevation of the enzyme activity in patients with acute hepatitis, chronic hepatitis, liver cirrhosis, malignant liver diseases and obstructive jaundice.

The results showed a mild increase in serum levels of 2'-Dcyd in patients with chronic liver diseases and 3-fold increase in patients with HCV. In the patients with HCC, 2'-Dcyd serum level was 8-fold higher than normal level. This agrees with the result of our previous study which revealed an increase of 2'-Dcyd level in serum with other malignancies (Abdellah *et al*., 2003).

We have studied the inflammatory marker (CRP) to determine its relation to 2'-Dcyd, to see if its elevation can be attributed to malignancy alone or to other inflammatory conditions. CRP was positive in more than 80% of HCC patients. But, In HCC group there was no significant difference between serum 2'-Dcyd levels between cases with negative or positive CRP. So, we can conclude that elevation of 2'-Dcyd is mainly attributed to malignancy and not to an inflammatory process. Similarly, no stati\stically significant difference was found between CRP positive and negative cases regarding levels of AFP, ALT and AST.

Cirrhosis was observed in 58% of HCC cases, 50% of CLD and only 20% of HCV cases. No significant difference in level of 2'-Dcyd between cirrhotic and non-cirrhotic cases in HCC group. On the other hand, AFP and liver enzymes were significantly higher in cirrhotic cases. Similar results were reported by Kane *et al*. (1997) who showed that chronic inflammatory status of liver frequently increases liver enzymes, AFP and production of nitric oxide. They also reported that patients with positive HCV infection who developed HCC on top of cirrhosis showed high levels of AFP and liver enzymes far exceeded those of liver cirrhosis alone.

In this study, no relation could be found between increased levels of 2'-Dcyd and cirrhosis or the inflammatory process. Chronic liver disease was not associated with a significant rise in 2'-Dcyd compared to control individuals. This illustrates that this marker is related to damage of hepatocytes rather than inflammation or chronic liver insult. Unfortunately, it could not be considered specific to HCC, as it was high enough in cases of HCV.

Using ROC curves, it is suggested to use 0.14 of 2'-Dcyd as a cut-off level for diagnosis of HCC. This level had a sensitivity of 93% and specificity of 90%. These levels were not higher than those of a AFP level of ≥ 3.55, which had a sensitivity of 95% and specificity of 100%. On the other hand, the same 2'-Dcyd level of 0.14 had a sensitivity and specificity of 90% for diagnosis of HCV. When HCV cases are added to control cases, specificity of this cut-off level drops to 50%.

We can conclude that for diagnosis of HCC, 2'-Dcyd is no better than AFP, as it is elevated in viral hepatitis C. A combination of AFP and 2'-Dcyd could provide broader information in diagnosis and treatment decision.

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قياس 2-دى أوكسى سيتدين كدليل بيولوجى لسرطان الكبد

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يعتبر 2-دى أوكسى سيتدين واحد من أربعة نيكلويوتيدات معروفة وموجودة فى سوائل الجسم المختلفة نتيجة لتحلل بعض الخلايا الميتة وتكسير الحمض النووى الدى أوكسى ريبوزى وتزداد نسبته فى حالات وجود أورام خبيثة. وأثبتت الدراسات السابقة أنه من الممكن استخدام ال 2-دى أوكسى سيدتين كدليل بيولوجى فى الكشف عن سرطان المثانة والليوكيميا والهدف من هذه الدراسة امتداداً للدراسات السابقة بحث إمكانية استخدام 2-دى أوكسى سيتدين كدليل بيولوجى للكشف عن سرطان الكبد وللوصول إلى هذا الهدف تم قياس 2-دى أوكسى سيتدين فى أربعة مجموعات مع مقارنة النتائج بدليل ثابت ومعروف وهو الألفا فيتوبروتين.

وهذه المجموعات هى:

مرضى سرطان الكبد (60 مريضاً)

مرضى أمراض الكبد المزمنة (20 مريضاً)

مرضى التهاب الكبد الوبائى (20 مريضاً)

المجموعة الضابطة وعددها 20 من الأفراد الأصحاء.

وأظهرت النتائج أن مستوى 2-دى أوكسى ستيدين يرتفع إلى ثمانية أضعاف فى مرضى سرطان الكبد ويرتفع ثلاث أضعاف فقط فى مرضى التهاب الكبد الوبائى بينما يرتفع مستوى 2-دى أوكسى سيتدين ارتفاعاً طفيفاً فى المجموعة التى تعانى من أمراض الكبد المزمنة بالمقارنة بالمجموعة الضابطة. كما أظهرت النتائج أن حساسية هذا الدليل 93% لمرضى سرطان الكبد و 90% لمرضى التهاب الكبد الوبائى عند قيمة مرجعية مقدارها 0.14 وهذه النتيجة ليس بأفضل من الألفافيتوبروتين ولكن من الممكن إذا جمعنا بين قياس 2-دى أوكسى سيتديين والألفافيتوبروتين الوصول إلى فكرة أفضل عن التشخيص ومن ثم اتخاذ قرار بدء العلاج مبكراً.