Hayvan ve İnsanlarda Hepatosellüler Karsinomun Erken Teşhisinde Alfa-L Fukosidaz'ın Alfa-fetoproteine olan Üstünlüğü

Priority of Alpha-L- Fucosidase Over Alpha fetoprotein in Early Detection of Hepatocellular Carcinoma in Animals and Human

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ÖZET

Amaç: Erken tanı hepatoselüler karsinom (HCC) tedavisindeki en kritik adımdır. Çalışmada erken tanıda alfa-L- fukosidazın (AFU) klasik belirteç olan alfa-fetoproteine (AFP) kıyasla güvenilirliği incelenmiştir.

Gereç ve Yöntem: Teorimizi kanıtlamak için çalışma hem insan hem de hayvan örneklemler kullanılacak şekilde planlanmıştır. Çalışmanın hayvan deneyi ayağında 60 Wistar faresi kontrol grubu ve 8, 16, 24 hafta dietilnitrozamine (DENA) verilen gruplar olmak üzere 4 gruba ayrılmıştır. İnsan çalışma grubu kontrol ve hafif, ileri ve metastatik olarak 4 gruba bölünen toplam 120 hastadan oluşmaktadır. Histolojik ve biyokimyasal analizler (AFP, AFU, karaciğer fonksiyon testleri, total antioksidan serum seviyeleri) ve ROC eğrileri ile korelasyon analizleri insan ve hayvan gruplarında yapılmıştır.

Bulgular: DENA-tedavi grubu zamana bağlı olarak belirgin histopatolojik değişiklikler göstermiştir. Kontrol grubu ile kıyaslandığında 6 hafta sonra bilirubin ve total antioksidanlar dışındaki tüm biyokimyasal parametrelerde, 8 hafta sonra bilirubin, albumin, AFP ve total antioksidanlar dışındaki parametrelerde ve 24 hafta sonra ise tüm biyokimyasal parametrelerde (AFP, AFU, karaciğer fonksiyon testleri ve total antioksidan serum düzeylerinde) anlamlı değişiklikler saptanmıştır. Histolojik olarak sınıflandırılmış tüm HCC gruplarının tün incelenen parametrelerinde istatistiksel olarak anlamlı sonuçlar saptanmıştır. Sınır olarak 5 µmol/l/min kabul edildiğinde ROC analizi ile yapılan incelemede AFU %90 sensitivite, %92 spesifite göstermiştir ve %91 tanısal doğruluk göstermiştir. Sınır değer 60 ng/ml olarak alındığında ise sensisivite, spesifite ve tanısal doğruluk sıraıyla %60, %76 ve %68 olarak saptanmıştır. AFU ile ALT, AST ve AFP ilişki katsayıları istatistiksel olarak anlamlıdır.

Sonuç: HCC erken tanısında erken tanıda AFU, AFP'den daha iyi bir belirteçtir. AFU sensitivite, spesivitesi daha yüksek olup diğer indekslerle AFP'ye kıyasla daha iyi korele olmuştur. İnsandan elde edilen sonuçlar teorimizi doğrulamıştır.

Anahtar Kelimeler: Hepatoselüler karsinom; Dietilnitrozamine; Alpha-L-fucosidase; Alpha-fetoprotein; Antioksidan seviyesi; Erken tanı

ABSTRACT

Objective: Early detection of hepatocellular carcinoma (HCC) is the most critical step in the management process. We try to provide new insights about the possible role of alpha-L- fucosidase (AFU) to achieve more reliable detection of HCC over a classical marker alpha fetoprotein (AFP).

Methods: The study included both animal and human subjects, to legislate our theory. In animal study, 60 Male Wistar rats were classified to control, other 3 groups received di ethyl nitrosamine (DENA) for 8, 16, 24 weeks respectively. In human study: one hundred and twenty patients were assigned as: control group, 3 groups with histologically graded HCC, into mild, advanced and metastatic HCC. Histological and biochemical analysis (AFP, AFU, liver function tests, total antioxidant serum levels) in both animals and patients, as well as, receiver operating characteristic (ROC) curves and correlation analysis, were executed.

Results: DENA-treated groups showed significant histopathological changes in a time dependant pattern. After 24 weeks, a significant variation in biochemical parameters (AFP,AFU, liver function tests and total anti-oxidant serum levels), after 6 weeks, a significant variations in all parameters except bilirubin and total antioxidants, after 8 weeks, a significant variations in all parameters except bilirubin, albumin, AFP and total antioxidants, compared to control group. All tested biochemical parameters showed non-significant correlation, compared to AFU.All histologically-graded human HCC groups showed a highly statistically significant variations in all tested biochemical parameters. ROC analysis showed that at a cut-off value of 5 µmol/l/min, AFU yielded a

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sensitivity and specificity of 90% and 92%, respectively producing diagnostic accuracy of 91%. While at a cut-off value of 60 ng/ml, AFP yielded a sensitivity and specificity of 60% and 76%, respectively producing diagnostic accuracy of 68%. Correlation coefficients between AFU versus ALT, AST and AFP were statistically significant

Conclusion: AFU is a more accurate marker than AFP for early diagnosis of HCC. AFU showed higher sensitivity, specificity and correlated more to other indices than AFP. Human data confirmed our theory.

Key Words: Hepatocellular carcinoma (HCC), Diethylnitrosoamine (DENA), Alpha-L-Fucosidase, Alpha-fetoprotein; Antioxidant status, Early detection

Introduction

Hepatocellular carcinoma (HCC) is a common malignancy, representing the fifth most common cancer worldwide with an incidence nearly equal to its death rate (1). Chronic infection by the hepatitis B and C viruses is the most common cause of HCC(2). Other important causes are cirrhosis, non-alcoholic steatohepatitis (NASH)(3), alcohol abuse, obesity, hemochromatosis, α1-antitripsin deficiency(4), and toxins as aflatoxin(5,6). The major clinical risk factor for the development of HCC is liver cirrhosis since 70–90% of HCCs develop on a cirrhotic liver (7). HCC is known to be a result of a large number of genetic and epigenetic alterations that are transformed to neoplastic stages. These alterations affect the proteins in certain major signaling pathways that control the cycle, proliferation, and cell survival (8).

HCC diagnosis is always discovered lately. In many patients, HCC is asymptomatic and is diagnosed in an advanced stage. This is why, surveillance is strongly recommended to detect early HCC to increase the chance for curative treatment and limit tumor-related death (9,10). Early detection of HCC is the most critical step in the management process. A combination of both pathological features and biochemical markers with high sensitivity and specificity is still main objective in medical practice(11). A number of serum markers have been proposed and currently used as an effective method for detecting HCC (12,13). The classical marker for HCC is AFP, however, it is not secreted in all cases of HCC(14). Alpha-L-fucosidase (AFU) is a lysosomal enzyme present in all mammalian cells. It has been proposed as a tumour marker since many studies reported increased AFU serum levels in patients with cirrhosis and HCC(15).

The goal of present study is to provide new insights about the possible role of AFU in early, accurate and sensitive detection of HCC. The study included both animal and human subjects, in a trial to legislate experimental observations. Histological and biochemical analysis in healthy and diseased subjects were executed. Moreover, ROC and correlation analysis were conducted.

Materials and methods

Experimental design

I.Animal study

The current study recruited 60 male Wistar rats (weighing 125-150 g at the beginning of experiment). They received diethylnitrosamine (DENA) in drinking water (100 mg/l,(16). DENA solution was kept in dark bottles since the molecule is light-sensitive and was prepared fresh every week. Animals were divided equally into 4 groups as follows:

Group-I: Fifteen rats were given sterile tap water, served as control.

Group-II: Fifteen rats were given DENA in drinking water (100 mg/l) for 4 weeks.

Group-III: Fifteen rats were given DENA in drinking water(100 mg/l) for 8 weeks.

Group-IV: Fifteen rats were given DENA in drinking water (100 mg/l)for 12 weeks.

One month after the end of DENA administration, all animals were scarified, serum was collected, frozen and livers were removed and stored in formalin for histopathological investigations, each group possessed 10 rats by the end of the experiment.

II- Human study

One hundred and twenty patients were recruited from Minia and Assiut National Cancer Institutes during one year period. Patients only with histologically proven HCC were included in this study after getting their approval. Patients were diagnosed according to radiological imaging, laboratory tests and clinical assessment, following the institutional protocol. Individual patient profiles were collected from records kept in the institutions. Table 3 presents the characteristics of the investigated groups. The study population was classified to the following groups:

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Group-I: Thirty healthy, age and sex-matched adult patients served as normal control.
Group-II: Thirty, age and sex-matched adult patients with histologically proven HCC patients with tumor size < 5 cm (mild HCC).
Group-III: Thirty, age and sex-matched adult patients with histologically proven HCC patients, with tumor size > 5 cm (advanced HCC).
Group-IV: Thirty, age and sex-matched adult patients with histologically proven HCC patients, having metastatic HCC.

Blood samples were collected, centrifuged, sera were collected and stored at –80°C. All samples were studied following the completion of the collection period; however, AFU activity was assayed within 30 days after collection(17). This step was taken to avoid any possible alterations in AFU activity after this period(18).

Histological Examination

For the histopathological study, rat liver specimens were taken5 mm away from the edge of the largest hepatic lobe, fixed with 10% formaldehyde; embedded in paraffin wax, stained with hematoxylin and eosin, then examined for histopathological changes.

Biochemical Analysis

AFU measurement

Serum AFU activity was assayed (19). Estimation of the serum levels and activities of other biochemical indices as, AFP, ALT, AST, total bilirubin, albumin and total antioxidants were carried out using commercial kits, following the instructions of the manufacturer.

Statistical analysis

Data were analysed using the Graph pad prism 6 statistical software. Data were expressed as mean value ± standard deviation. Significant difference between groups was analyzed by t-student test. The relationship between continuous variables was analyzed by Pearson's correlation coefficient. Sensitivity, specificity and diagnostic accuracy of AFP and AFU were calculated from the corresponding (ROC) curves. The optimal cut-off value was set as the nearest point to the upper left corner of the curve (the highest point of the plot)(20)

Results

I-Animal study

Histopathological examination in DENA group, given for two weeks, showed peri portal inflammatory infiltration and few fibroblastic proliferation associated with hyperplasia and dilatation in the bile ducts and formation of new one while the heptocytes had degenerative change (Figure 1C,1D), in comparison to control animals (Figure 1A,1B). After 8weeks, hepatocytes showed ballooning degeneration, associated with congestion in the portal vein, inflammatory cells infiltration and periductal fibrosis with oedema in the portal area (Figure 1E,1F) and fibrosis ,associated with new formed bile ductules (Figure 1G,1H). The bile ducts showed multiple number of bile ductules with hyper chromatic nuclei as well as different ratio between the cytoplasm and nucleus and cholingiocarcinoma (Figure 1I,1J,1K). After 12 weeks, these features were aggravated with appearance of congestion in the central vein (Figure 1L).

Biochemical analysis

AFU besides other tested biochemical markers showed time-dependent variations correlated with DENA treatment duration (8, 16 and 24 weeks). DENA-treated group (24 weeks) showed statistically significant variations in all tested parameters (AFP, AFU, liver function tests, total anti-oxidants serum levels), DENA-treated group (16 weeks) showed significant variations in all parameters except bilirubin and total antioxidants, while after8 weeks, a significant variation in all parameters except bilirubin, albumin, AFP and total antioxidants, were observed (Table 1). All tested biochemical parameters showed non-significant correlation coefficients in relation to AFU (Table 2, Fig 4A).

II-Human study

Demographic patient profiling

Table 3 summarizes characteristics of patients (i.e, age, sex, liver cirrhosis, hepatitis B and C viral infections, diabetes mellitus, deep venous thrombosis and hypothyroidism).

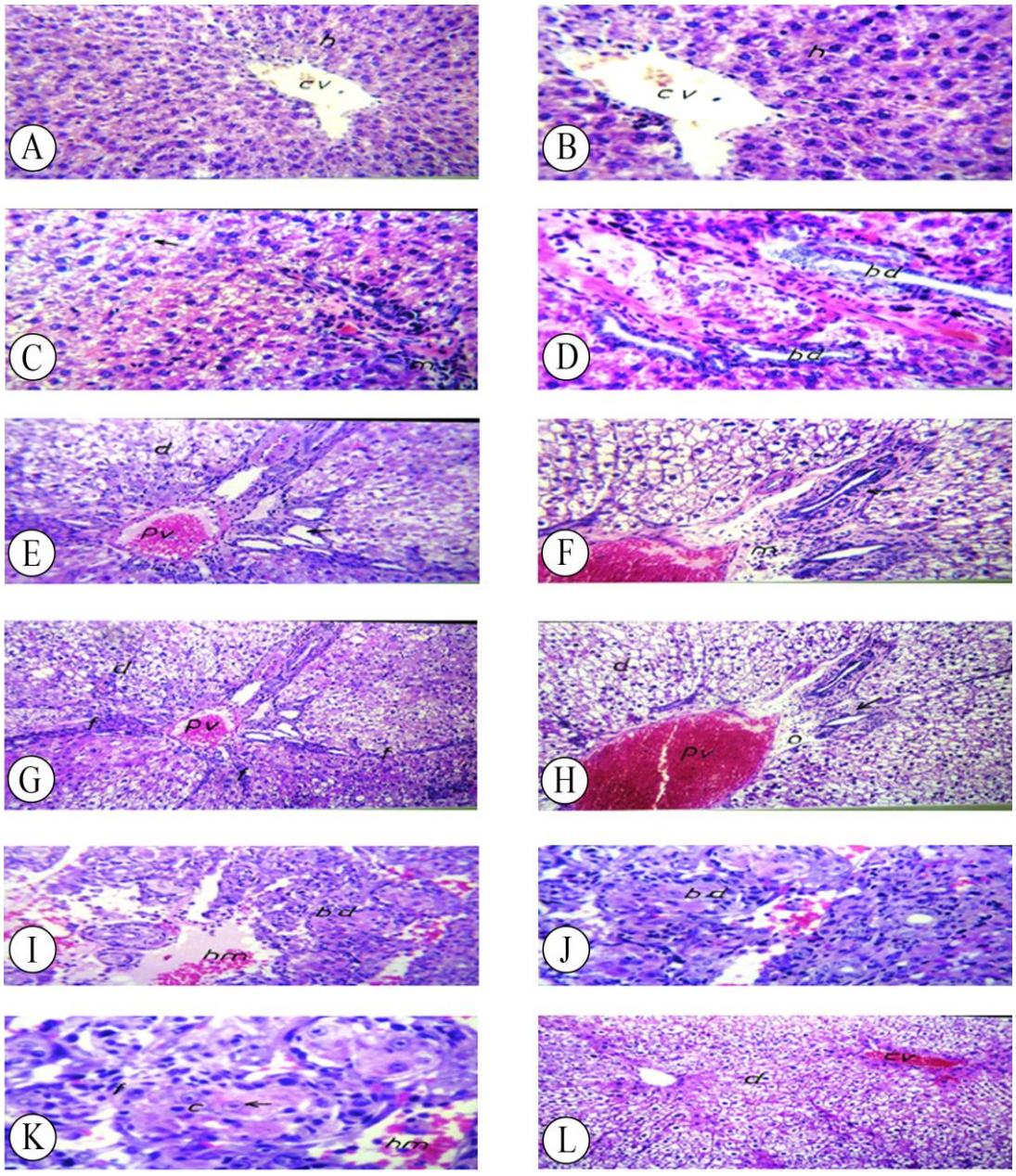
Biochemical analysis

AFU besides other tested biochemical markers showed time-dependent variations correlated with stages of HCC (mild, advanced and metastatic HCC). All histologically-proven HCC groups showed highly statistically significant variations in all tested biochemical parameters (AFP, AFU, liver function tests, total anti-oxidants serum levels, Table 4). Correlation coefficients of AFP, ALT, and AST versus AFU were statistically significant (Table 5, Fig 4B).

ROC analysis in the selected human subjects

At a cut-off value of 5 µmol/L/min, AFU sensitivity and specificity were 90% and 92%, respectively producing diagnostic accuracy of 91%. While at a cut-off value of 60 ng/ml, AFP sensitivity and specificity were 60% and 76%, respectively producing diagnostic accuracy of 68%. This reflects higher specificity and sensitivity of AFU over AFP in different disease stages (Table 6, Figure 2,3).

Figure 1: Histological examination of liver sections from different groups in DENA intoxicated groups, compared to normal rats:



Group I: Normal histopathological picture o central vein (cv) and surrounding hepatocytes (A, B). Group II: Inflammatory cellular infiltration and proliferation with hyperplasia, the bile ducts dilatation in and hepatocytes degenerative change (C,D). Group III: Ballooning degeneration in the hepatocytes with congestion in the portal vein (PV), inflammatory cell infiltration and periductal fibrosis with inflammatory cell infiltration (E, F). Fibrosis was extended in between the hepatocytes associated with new formed bile ductules (G, H). The bile ducts showed anaplastic alteration characterized by multiple number of ductules with pleomorphic lining epithelium and hyperchromatic nuclei as well as different ratios between the cytoplasm and nucleus (I, J, K). Ballooning degeneration in the hepatocytes with congestion in the central vein were observed (L).

Table 1: Variations in liver function tests, total antioxidants, AFP and AFU in DENA intoxicated groups, compared to normal rats (Values are expressed as mean± SD, n=10)

Parameter / Period	Control	4 Weeks	8 Weeks	12 Weeks
ALT (U/L)	2.6 ± 0.3	14.7 ± 0.1***	21.6 ± 2.6***	32.71 ± 2.8***
AST (U/mL)	33.6 ± 1.4	123.6 ± 4.7***	149.3 ± 12.4***	256.4 ± 28.3***
Bilirubin (mg/dl)	0.31 ± 0.03	0.35 ± 0.05	0.3 ± 0.06	0.6 ± 0.1**
Albumin (g/dl)	3.6 ± 0.1	3.5 ± 0.23	3.1 ± 0.08**	2.2 ± 0.2***
Total antioxidants(µm/L)	2.1 ± 0.13	1.7 ± 0.1	1.8 ± 0.17	1.6 ± 0.08**
AFP (ng/ml)	24.2 ± 1.1	27.30 ± 2	102.9 ± 10***	260.5 ± 21.2***
AFU (µmol/L/min)	0.16 ± 0.02	0.25 ± 0.02**	0.5 ± 0.02***	0.6 ± 0.04***
Levels of significant change from control,(*** P < 0.001: highly significant, ** P < 0.01: significant, * P < 0.05: mildly significant.				

Table 2: Correlation coefficients for different parameters compared to AFU in rats

Pearson r	AFP	ALT	AST	Bilirubin	Albumin	Total antioxidants
r	0.88	0.95	0.9	0.72	- 0.88	- 0.64
95% CI	- 0.52 to 0.99	- 0.15 to 0.99	- 0.43 to 0.99	- 0.795 to 0.99	- 0.99 to 0.53	- 0.99 to 0.84
R squared	0.78	0.9	0.82	0.52	0.77	0.41
P (two-tailed)	0.12	0.052	0.095	0.28	0.12	0.34
P value	ns	ns	ns	ns	ns	ns
Significant? (alpha = 0.05)	No	No	No	No	No	No
CI; Confidence Interval						

Table 3: Demographic patient data used in the human study

% Suffering / Complaint	Mild HCC	Advanced HCC	Metastatic HCC
Liver Cirrhosis	5.88	60	10
Positive HCV	5.88	30	0
Positive HBV	11.76	10	0
Diabetes Mellitus	23.52	10	10
History of Deep Venous Thrombosis (DVT)	5.88	0	0
Hypothyroidism	0	10	0

Table 4: Variations in liver function tests, total antioxidants, AFP and AFU in patient groups, compared to normal individuals (Values are expressed as mean± SD, n=30)

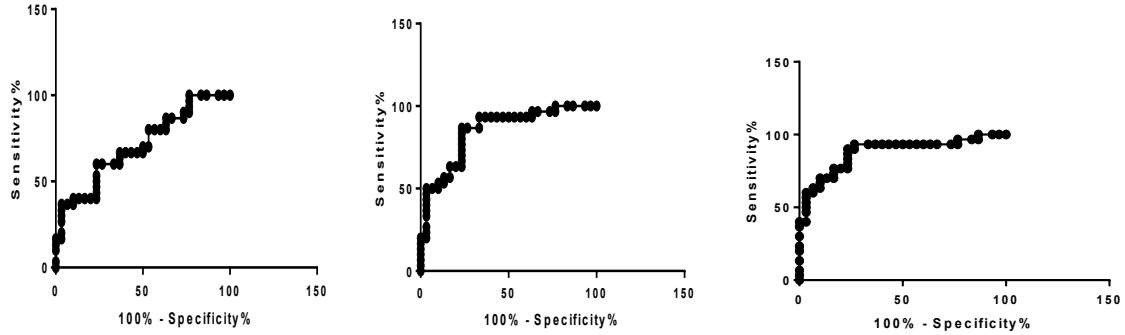
	Normal Control	Mild HCC	Advanced HCC	Metastatic HCC
ALT (U/L)	17.2 ± 2.1	52.8 ± 6.5 ***	76.4 ± 12.3 ***	96.9 ± 19.8 ***
AST (U/mL)	17.7 ± 1.7	94.3 ± 19.2 ***	124.9 ± 19.3 ***	139.4 ± 27 ***
Bilirubin (mg/dL)	8.06 ± 1.5	23.0 ± 3.4 ***	25.01 ± 3.9 ***	44.7 ± 7.2 ***
Albumin (g/dL)	41.6 ± 1.4	11.05 ± 3.3 ***	20.07 ± 3.4 ***	11.13 ± 3.4 ***
Total antioxidants(µm/L)	3.7 ± 0.5	2.1 ± 0.2 **	2.2 ± 0.15 **	1.5 ± 0.25 ***
AFP (ng/mL)	56.0 ± 9.3	113.4 ± 17.5 **	144.7 ± 14.7 ***	208.1 ± 24.2 ***
AFU (µmol/L/min)	2.2 ± 0.4	6.4 ± 0.5 ***	11.56 ± 1. 1 ***	13. 9 ± 1.2 ***
Levels of significant change from control, (*** P< 0.001: highly significant, ** P < 0.01: significant, < 0.05: mildly significant).				

Table 5: Correlation coefficients for different parameters compared to AFU in the studied patient

Pearson r	AFP	ALT	AST	Bilirubin	Albumin	Total antioxidants
R value	0.97	0.99	0.96	0.92	-0.75	-0.88
95% confidence interval	0.12 to 0.99	0.67 to 0.99	-0.01 to 0.99	-0.37 to 0.99	-0.99 to 0.76	-0.99 to 0.52
R squared	0.94	0.98	0.92	0.84	0.56	0.78
P (two-tailed)	0.03	0.01	0.04	0.08	0.25	0.12
P value	*	**	*	ns	ns	ns
Significant ? (alpha = 0.05)	Yes	Yes	Yes	No	No	No

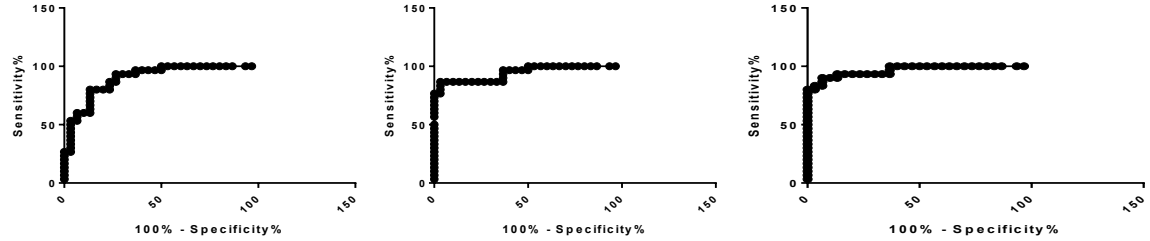
Table 6: Sensitivity, specificity and diagnostic accuracy of AFP and AFU at different cut-off values among studied patients (Underlined values represent the optimal cut-off value calculated from the ROC curve)

	Cut-off Values	Sensitivity %	Specificity%	Diagnostic accuracy%
AFP (ng/mL)	20	90	24	57
	80	36	58	58
	60	60	76	68
	100	40	76	58
	200	26	96	58
AFU (µmol/L/min)	2	96	54	75
	4	80	86	83
	5	90	92	91
	10	76	100	88
	15	40	100	70



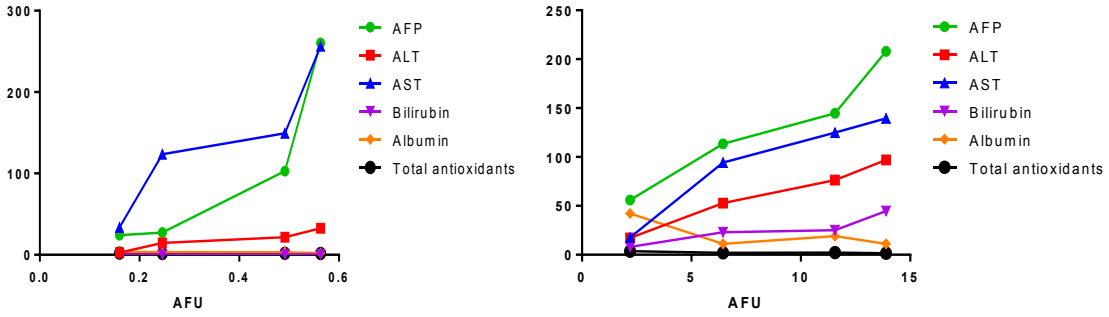
A B C

Figure 2: A: Receiver Operating Characteristic (ROC) curve for AFP levels in mild HCC, area under the curve (Diagnostic efficacy index) = 0.71. B: ROC curve for AFP levels in advanced HCC area under the curve (Diagnostic efficacy index) = 0.85. C: ROC curve for AFP levels in metastatic HCC, area under the curve (Diagnostic efficacy index) = 0.88.



A B C

Figure 3: A: ROC curve for AFU activities in mild HCC, area under the curve (Diagnostic efficacy index) = 0.9, B: for AFU activities in advanced HCC, area under the curve (Diagnostic efficacy index) = 0.9, C: for AFU activities in metastatic HCC, area under the curve (Diagnostic efficacy index) = 0.96.



A B

Figure 4: A: Correlation between AFU versus different parameters (Animal Study). B: Correlation between AFU versus different parameters (Human Study).

Discussion

AFU is a glycosidase primarily found in lysosomes and involved in the degradation of a variety of fucose-containing fucoglyco-conjugates (21). The alterations of AFU catalytic activity in human tissues and body fluids have a diagnostic value for human tumors including primary HCC besides other tumors as colorectal and ovarian cancers (22,23). The deficiency of AFU activity in female sera is probably a hereditary condition related to higher risk of ovarian cancer. The persistently elevated AFU level in sera of

patients with liver cirrhosis contributes to early detection of HCC (21,24).

The current gold standard and most commonly used biomarker for patients at risk for HCC is AFP. AFP along with ultrasound every 6 to 12 months, despite being the most applicable tool for HCC diagnosis, yet proved to have serious limitations (25-27).Some reports have indicated that the high serum concentration of AFP correlates with the poor prognosis of HCC patients (28). However, two thirds of HCC patients with the nodule less than 4 cm have serum AFP levels less than 200 ng/mL and up to 20% HCC patients do not produce AFP. Moreover, it has limited utility of differentiating HCC from benign hepatic disorders for the high false-positive and false-negative rates (24). AFP is negative in approximately 40% patients with early stage HCC. Even in advanced HCC, the level of AFP may be normal in 15-30% of patients (11). Ultrasound surveillance even performed at every three monthly intervals cannot improve detection of small HCC because of limitations in recall procedures (29).

That is why the need for other reliable serum markers for HCC has become at the highest priority. In this study, AFU serum levels showed time-dependent variations correlated with DENA treatment duration in animals or different progressive stages of HCC in humans. Controversial views about AFU have been reported. In 1984, first reported that AFU is overexpressed in patients with HCC liver changes (30). It has been proved that the values of AFU serum concentration were not correlated with the tumor size and were frequent in early HCC cases (30). Other studies showed that the sensitivity and specificity of AFU for diagnosis of primary HCC were 70-80% (18,31). In contrast to AFP, the activity levels of AFU were not correlated with tumor magnitude and AFU was of value in the diagnosis of HCC patients with negative or low serum levels of AFP, particularly for small HCC (<5 cm) (24, 32-34).

El-Housseini et al. demonstrated that the sensitivity of AFP in tumor detection was 68.2% (31). This level of detection was increased to 88.6% when AFP was evaluated in conjunction with AFU. Other recent study showed that the sensitivity of AFU for HCC was only 56.6% which was lower than previous reports, owing to a higher cut-off value (636.5 µmol/L/h) with a higher diagnostic specificity (82.4%). To improve the diagnostic sensitivity, the combined detection of AFU with other tumor markers should be commonly used in clinical practice (35).

Conclusion

AFU is more valuable than AFP as an accurate tumour marker for early diagnosis of HCC. Human data seem to be more consistent and reflective for disease progression than animal data. AFU showed higher sensitivity, specificity and diagnostic accuracy than AFP. It also showed significant correlation coefficients with the classical markers for liver diseases (AFP, ALT and AST) at different stages which constitutes a solid panel for HCC diagnosis. The application of the theory on human subjects in the same study, legalised the use of AFU as a more promising tumor marker in early speculation of HCC among risky individuals.

Conflict of interest: None**.**

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