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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(1): 01-10. **OPEN ACCESS**

Correlative Study of Some Tumor Markers in Patients with Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) the sixth most common cancer and the second cause of cancer-related death worldwide. AFP, AFP-L3 and GPC-3 have been proposed as markers for early detection of HCC. Objective: to investigate the diagnostic value of serum GPC3 compared to AFP and AFP L3 as a diagnostic biomarker of HCC. Subjects and Methods: A total of 112 subjects, 62 HCC patients and 50 compensated cirrhotic, were studied. An enzyme-linked immunosorbent assay (ELISA) was used for the quantitative and sensitive determination of serum AFP, APF-L3 and GPC3 levels. Results: In HCC patients, AFP (ng/ml), AFPL3 (ng/ml) and GPC3 (ng/ml) levels and combined of AFP and APF-L3 with GPC3 (AAG score) were extremely significantly higher ($p < 0.0001$) than in compensated cirrhotic patients. They were elevated in large tumor and tumor types with significant difference ($p < 0.05$) for AFP and AFPL3 ($p < 0.0001$and $p < 0.001$) for GPC3 and AAG score compared with small tumor and unifocal tumor types; respectively. Serum GPC3 was more sensitive and specific than AFP and AFP L3; and combination of GPC3, AFP and AFP-L3 in AAG score was become more accurate than any studied marker alone. Conclusions: GPC3 is acceptable as a serum marker for the diagnosis of HCC, which can elevate the accuracy of diagnosis. The combination of GPC3 with AFP and AFP L3 could improve the diagnostic sensitivity for HCC. Thus, signatures of a combination of biomarkers may be more valuable for the diagnosis, staging, and prognosis of HCC.

Keywords: Early diagnosis, HCC, AFP, AFP-L3, GPC3, biomarker

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and represents sixth most common cancer worldwide and the second cause of cancer-related death worldwide (Ziada et al., 2016). HCC is a silent disease, showing no symptoms in the early stages, so, the majority of patients are diagnosed with advanced disease (Brito et al., 2016). Therefore, a diagnosis must be made sufficiently early to perform curative and effective treatments (Niu et al., 2016). Currently, detection of HCC in clinical practice is performed by diagnostic imaging techniques and determination of serum biomarkers. However, these methods display limitations in sensitivity and specificity, especially with respect to early stages of HCC (Reichl and Mikulits, 2016). Although, ultrasonography is the most widely used imaging for HCC screening because of its diagnostic accuracy, noninvasiveness, good acceptance by patients, and moderate cost (McKillop and Schrum, 2009), it is highly dependent on the operator's experience. Measuring tumor biomarkers levels for HCC is an important tool for

disease management. There are many categories of biochemical markers that are being used or studied for the detection of HCC: Oncofetal antigens, glycoprotein antigens, enzymes and isoenzymes (Zhou et al., 2010). Alpha fetoprotein (AFP), alpha fetoprotein L3 (AFP-L3) and glypican 3 (GPC3) have been established as HCC-specific tumor markers (Toyoda et al., 2015). AFP is large serum glycoprotein, belonging to onco-development protein. AFP used in differential diagnosis and follow-up of patients with liver tumors (AlSalloom, 2016). Moreover, total AFP can be divided into three different glycoforms (L1, L2 and L3) according to their binding capacity for lens culinaris agglutinin (LCA) or their isoelectric point difference (Kobayashi et al., 2016). Moreover, Kandil and Cooper, (2009) found that glypicans interact with growth factors and modulate their activities; hence, they play an important role in cell growth, differentiation and migration. As an oncofetal antigen, GPC3 is a useful molecular marker for HCC diagnosis, especially in poorly-differentiated or small HCC (Iglesias et al., 2008). The identification of more than one biomarker appears to enhance their individual performance and diagnostic accuracy for HCC (Juárez-Hernández et al., 2017). Thus, the aim of the current study was to determine the diagnostic performance of these biomarkers for the detection of HCC by comparing the sensitivity and specificity of each biomarker alone and in combination among HCC patients and compensated cirrhotic patients. In addition, determine the diagnostic performance of them for differentiate tumor size and type.

MATERIALS AND METHODS

The present study was conducted on 112 subjects admitted to Mansoura University Hospitals, Mansoura, Egypt from September 2015 to May 2017. Patients with hereditary or systemic disorders were excluded. They were grouped into 62 patients with HCC (50 males and 12 females) with mean age 50.0±5.6 ranged 40 – 64 years and 50 patients with compensated liver cirrhosis (32 males and 18 female) with mean age; 49.6±5.6 ranged 41 – 61 years. The diagnosis of HCC in patients was carried out according to the American Association for the Study of Liver Diseases (AASLD) Practice Guidelines. HCC diagnosis depend on presence of hepatic focal lesion (s) detected by liver ultrasound (US), and confirmed by triphasic computed tomography (CT) scan and/or dynamic magnetic resonance imaging (MRI) techniques. None of the HCC patients had received transarterial embolization or chemotherapy or underwent radiofrequency ablation or surgical interference. Blood samples were drawn from patients and divided into two tubes; first tube containing sodium citrate for assaying INR. Second tube (plan) was centrifuged for 10 minute at 4000 rpm and the sera were pipetted and kept frozen at -20 ºC till used. HCC patients were classified according to tumor type and size is shown in Table 1.

Methods

Biochemical and hematological tests

Liver function tests alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin and prothrombin time were measured using Human assay kits (Human Company for biochemical, 65205 Wiesbaden, Germany).

Quantitatively determination of serum AFP, AFP-L3, GPC3

Serum AFP, AFP-L3 and GPC3 was quantitatively determined by using ELISA kits. AFP and AFP-L3 kits were supplied by UBI MAGIWELL quantitative kits (USA) and GPC3 kit was supplied by Bio Mosaics Co. Burlington, VT 05405, USA.

Statistical analysis

Statistical analyses were performed by SPSS software version 22.0 (SPSS Inc., Chicago, IL). Continuous variables were expressed as mean ± standard deviation (SD). Statistically significant differences were determined using ANOVA, Student t test and Mann-Whitney U test. Statistically, value of $P > 0.05$ is considered not significant, $P < 0.05$ is considered significant and $P < 0.001$ is considered highly significant. Stepwise linear regression analysis was used to develop the best HCC diagnostic HCC score [AAG score = 1.0 + (0.0001 x AFP) + (0.001 x AFPL3) + (0.1 x GPC3)]. The result showed that the original regression coefficients were not estimable, so that a simplified score was calculated by summing up the single markers.

The diagnostic value was estimated by calculating the area under the receiver operating characteristic curves. Based on the receiver-operating characteristic analysis, the best cutoff points were selected and diagnostic performances sensitivity and specificity were determined.

RESULTS

Laboratory

charactrestics of the studied groups

Table 1 showed that**,** there were significant elevations of serum AST and ALT enzymes activities and total bilirubin level and prothrombin-INR ($p < 0.0001$) in HCC patients compared with compensated cirrhotic patients. While, there was significant decrease of serum albumin level in HCC patients compared with compensated cirrhotic patients.

Evaluation of serum AFP, AFP-L3, GPC3 and AAG score in patients with HCC comperd without HCC

As shown in **table 2**, in HCC patients serum AFP (ng/ml), AFPL3 (ng/ml), GPC3 (ng/ml) levels and AAG score were 358.4±182.1, 15.6±3.2, 11.2±3.6 and 2.2±0.36 significantly higher than compensated cirrhotic patients that had 46.2±10.7, 6.3±.3.0, 2.8±1.5 and 1.3±0.15 respectively with $p < 0.0001$for all.

Table 1: Patient Characteristics

Parameter	Compensated Cirrhosis (n=50)	HCC (n=62)	*P value*
Gender:			
Male	32 (64.0%)	50 (80.6%)	-
Female	18 (36.0%)	12 (19.4%)	-
Age:			
Mean ±SD, years	49.6±5.6	50.0±5.6	> 0.05
Range, years	41 - 61	40 - 64	-
Tumor size:			
Very small, less than 2 cm	N/A	0 (0.0%)	-
Small size, 1 HCC or 3 nodules less than 3 cm	N/A	32 (51.5%)	-
Multinodular with no pv thrombosis	N/A	15 (16.1%)	-
Advanced with pv invasion	N/A	17 (27.4%)	-
Tumor type:			
Unifocal	N/A	36 (58.1%)	-
Other	N/A	26 (41.9%)	-
AST (U/L)	53.8±15.0	40.7±9.9	< 0.0001
ALT (U/L)	64.8±18.7	47.5±11.4	< 0.0001
T. bilirubin (mg/dL)	1.85±0.86	2.40±0.49	< 0.0001
S. Albumin (g/dL)	3.38±0.58	2.69±0.25	< 0.0001
INR	1.35±0.37	1.84±0.22	< 0.0001

Data are presented as means ± Standard division**.** HCC: Hepatocellular Carcinoma. As shown in table (2): the most common HCC type is unifocal and the most common size is the second stage in Milan criteria (early stage, 1 HCC or 3 nodules less than 3cm.

Table 2: Evaluation of serum AFP, AFP-L3, GPC3 and AAG score

Patients	AFP (ng/ml)	AFPL3 (ng/ml)	GPC3 (ng/ml)	AAG score
Studied groups				
Compensated Cirrhosis	46.2±10.7	6.3±.3.0	2.8±1.5	1.3±0.15
HCC	358.4±182.1	15.6±3.2	11.2±3.6	2.2±0.36
P value	***< 0.0001******	***< 0.0001******	***< 0.0001******	***< 0.0001******
Tumor size				
Small size	351.0±121.0	15.2±2.7	9.3±2.7	1.9±0.27
Large size	367.8±245.4	16.0±3.7	13.9±2.9	2.4±0.30
P value	***< 0.05***	***< 0.05***	***< 0.0001******	***< 0.0001******
Tumor type				
Unifocal type	379.0±116.4	15.2±2.7	9.9±2.9	2.0±0.29
Other types	329.1±245.9	16.2±3.7	13.0±3.8	2.4±0.39
P value	***< 0.05***	***< 0.05***	***< 0.001****	***< 0.001****

Data are presented as means ± Standard division. **P:** probability; P > 0.05 considered not significant, P < 0.05 considered

significant, *P < 0.01 considered high significant, **P < 0.001 considered very high significant and ***P < 0.0001 considered extremely significant.

Evaluation of serum AFP, AFP-L3, GPC3 and AAG score according to tumer size

As shown in **table 2,** according to tumor size, serum AFP, AFP-L3 GPC3 levels (ng/ml) and AAG score in HCC patients with large tumor size were 367.8±245.4, 16.0±3.7, 13.9±2.9 and 2.4±0.30 significantly higher than 351.0±121.0; p > 0.05, 15.2±2.7; p > 0.05, 9.3±2.7; p < 0.0001 and 1.9±0.27; p < 0.0001 in HCC patients with small tumor size, respectively**.**

Evaluation of serum AFP, AFP-L3, GPC3 and AAG score according to tumer type

As shown in table 2 according to tumor type, serum AFP, AFP-L3 and GPC3 levels (ng/ml) and AAG score were 379.0±116.4, 15.2±2.7, 9.9±2.9 and 2.0±0.29 in HCC patients with unifocal tumor type significantly lower than 329.1±245.9; p < 0.05, 16.2±3.7; p < 0.05, 13.0±3.8; p < 0.001 and 2.4±0.39; p < 0.001 in other tumor types.

Diagnostic Performance of serum AFP, AFP-L3, GPC3 and AAG score

As shown in table 3 and figure 1A, using ROC curve for differentiate HCC from compensated cirrhosis, serum GPC3 at 5.2 ng/mL yielded AUC = 0.986 with sensitivity 96.8%, specificity 92.0%, PPV 93.8%, NPV 95.8% and efficiency 94.6% more accurate of AFP at cut-off of 200.0 ng/mL and AFPL3 at cut-off 11.0 ng/mL yielded AUC = 0.902 and AUC = 0.956; with sensitivity 80.6%, 90.3%, specificity 100.0%, 92.0%, PPV 83.7%, 93.3%, NPV 80.6% 88.5% and efficiency 89.3%, 91.0%; respectively. Moreover, combination of AFP, AFP-L3 and GPC3 (AAG score) = 1.0 + (0.0001 x AFP) + (0.001 x AFPL3) + (0.1 x GPC3) at cut-off was 1.5 yield AUC = 0.990 with sensitivity 98.4%, specificity 92.0%, PPV 93.8%, NPV 97.8% and efficiency 95.5% more accurate any marker alone for differentiate HCC from compensated cirrhosis.

Moreover, as shown in table 3 and figure 1B, using ROC curve for differentiate of HCC patients with small tumor size from those large tumor size, serum GPC3 at 12.5 ng/mL yield AUC = 0.869 with sensitivity 75.4%, specificity 83.3%, PPV 74.0%, NPV 77.0% and efficiency 75.8% more sensitive and specific of AFP at cut-off of 400.0 ng/mL yield AUC = 0.573; with sensitivity 53.8%, specificity 66.7%, PPV 53.8%, NPV 66.6% and efficiency 66.7%, and serum AFPL3 at cut-off 16.7 ng/mL yield AUC = 0.659 with sensitivity 57.7%, specificity 75.0%, PPV 62.5%, NPV 71.0% and efficiency 67.7%. While, AAG score at cut-off was 2.2 yield AUC = 0.870 with sensitivity 77.0%, specificity 83.3%, PPV 77.0%, NPV 83.3% and efficiency 80.6% more accurate any marker alone for diagnosis of HCC patients with small tumor from large tumor size.

Table 3: Diagnostic performance of serum AFP, AFP-L3, GPC3 and AAG score

Marker	Area under curve	Cut-off	Sensitivity	Specificity	PPV	NPV	Eff.
Differentiate HCC from Compensated Cirrhosis							
AFP	0.902	200.0	80.6%	100.0%	83.7%	80.6%	89.3%
AFPL3	0.956	11.0	90.3%	92.0%	93.3%	88.5%	91.0%
GPC3	0.986	5.2	96.8%	92.0%	93.8%	95.8%	94.6%
AAG score	0.990	1.5	98.4%	92.0%	93.8%	97.8%	95.5%
Differentiate tumor size (Large size vs Small size)							
AFP	0.573	400.0	53.8%	66.7%	53.8%	66.6%	66.7%
AFPL3	0.659	16.7	57.7%	75.0%	62.5%	71.0%	67.7%
GPC3	0.869	12.5	75.4%	83.3%	74.0%	77.0%	75.8%
AAG score	0.870	2.2	77.0%	83.3%	77.0%	83.3%	80.6%
Differentiate tumor type (Unifocal type vs Other types)							
AFP	0.481	400.0	46.2%	61.1%	46.2%	61.1%	54.8%
AFPL3	0.690	16.7	61.5%	64.0%	55.2%	70.0%	63.0%
GPC3	0.710	12.5	69.2%	72.2%	64.3%	76.5%	71.0%
AAG score	0.760	2.2	73.1%,	75.0%	70.4%	80.0%	75.8%

AAG score = 1.0 + 0.0001 x AFP + 0.001 x AFPL3 + 0.1 x GPC3

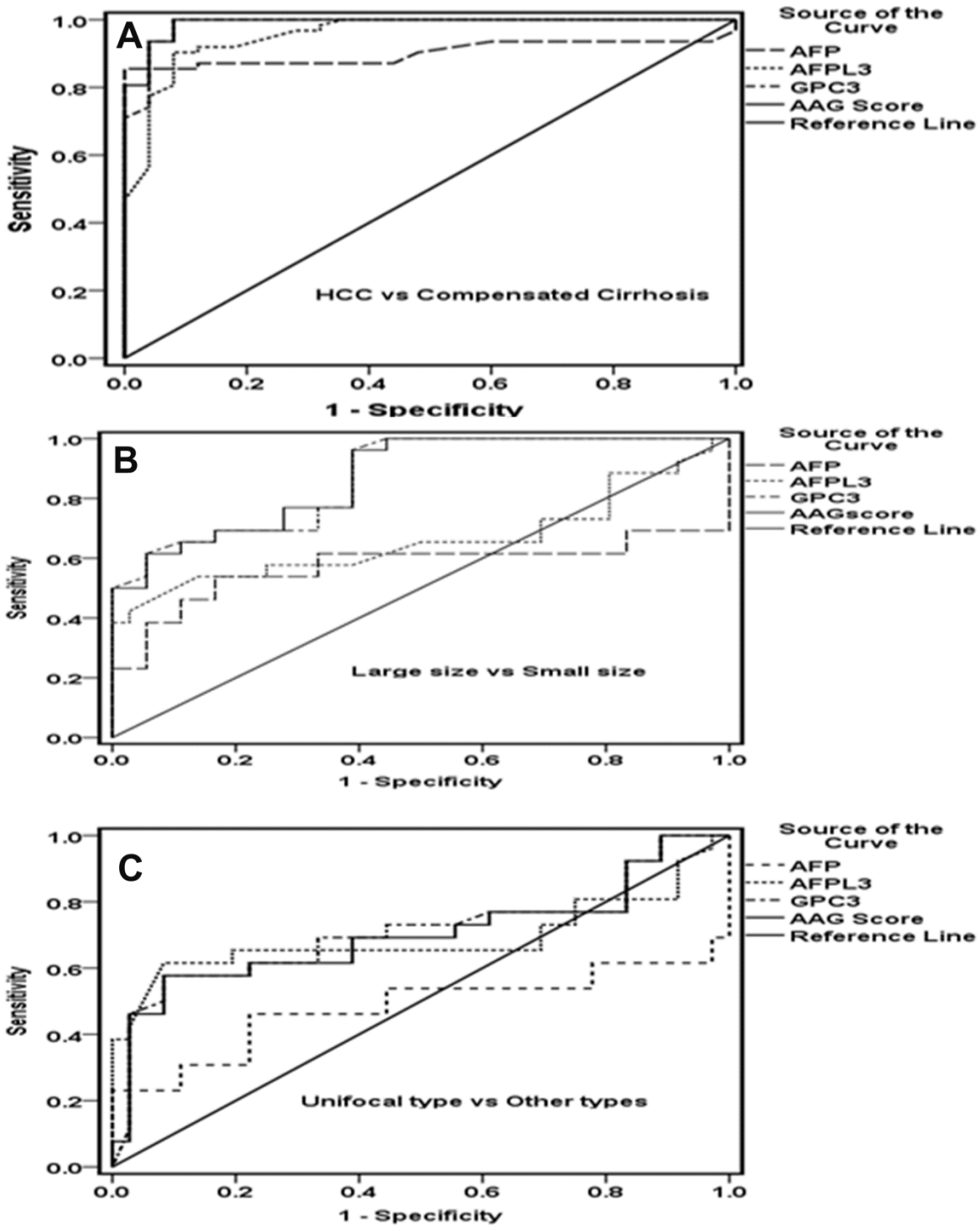

Figure 1: Receiver-operating characteristic (ROC) curve of serum AFP, AFP-L3, GPC3 and AAG score. (A) Differentiate HCC from Compensated Cirrhosis. **(B)** Differentiate tumor size (Large size vs Small size). **(C)** Differentiate tumor type (Unifocal type vs other types).

In addition, as shown in table 3 and figure 1C, for the diagnosis of HCC patients with unifocal tumor type from those HCC patients with other tumor types, serum GPC3 at 12.5 ng/mL yield AUC = 0.710 with sensitivity 69.2%, specificity 72.2%, PPV 64.3%, NPV 76.5% and efficiency 71.0% more sensitive and specific of AFP at cut-off of 400.0 ng/mL yield AUC = 0.481; with sensitivity 46.2%, specificity 61.1%, PPV 46.2%, NPV 61.1% and efficiency 54.8%, and serum AFPL3 at cut-off 16.7 ng/mL yield AUC = 0.690 with sensitivity 61.5%, specificity 64.0%, PPV 55.2%, NPV 70.0% and efficiency 63.0%. While, AAG score, at cut-off was 2.2 yield AUC = 0.760 with sensitivity 73.1%, specificity 75.0%, PPV 70.4%, NPV 80.0% and efficiency 75.8% more accurate any marker alone for the diagnosis of HCC patients tumor type.

DISCUSSION

Effective biomarkers (especially blood biomarkers) have a lower economic burden, do not require invasive methods, and are easy to obtain (Yu et al., 2016). Serum AFP is the most widely used biomarker in HCC surveillance programs and, until recently, was included in international guidelines for HCC surveillance (Bird et al., 2016). While, Tsuchiya et al., (2015) found some patients with cirrhosis and/or hepatic inflammation can have an elevated AFP, even without the presence of a tumor. In order to significantly improve the diagnostic accuracy for HCC, additional biomarkers are needed to complement AFP, especially due to the fact that many patients with benign liver diseases, such as chronic hepatitis, liver cirrhosis and gastrointestinal cancer, also have elevated serum AFP. The oncofetal antigen GPC3 is a glycosylphosphatidyl in ositolanchored membrane protein and has been shown to be present in sera from 40 to 50% of HCC patients (Iwama et al., 2016). In the present study, our results showed that serum AFP, AFP-L3 and GPC3 levels were significantly elevated in HCC patients more than compensated cirrhotic patients; $p < 0.0001$ for all. Agreed with Pontisso *et al.,* (2006) when found, AFP level increase in HCC patients due to the re-expression of the related gene, which is usually repressed in adult subjects. Durazo et al., (2008) studied 144 with HCC, 47 with chronic hepatitis and 49 with cirrhosis and found levels of AFP and AFP L-3 were significantly higher in patients with HCC than in those without HCC ($P < 0.0001$). Chen et al., (2013) measured serum GPC3 in a total of 1037 subjects, including 155 patients with HCC, 180 with chronic hepatitis, 124 with liver cirrhosis, 442 with non-HCC cancer and 136 healthy controls. They found that, the average level of serum GPC3 (ng/mL) in HCC patients was 99.94±267.2, which was significantly higher than in patients with chronic hepatitis 10.45 ± 46.02, liver cirrhosis 19.44 ±50.88, non-HCC cancer 20.50 ± 98.33 and healthy controls 4.14 ± 31.65. These findings are often difficult to diagnose by various imaging modalities in small HCCs. Such blood supply changes typically result in change of echo pattern in nodules. In the present study, serum AFP, AFP-L3 and Glypican-3 and AAG score were elevated in large tumor with no significant difference ($p < 0.05$) for AFP and AFP L3 but with significant difference ($p < 0.0001$) for GPC3 and AAG score compared with small tumor and also, they elevated in tumor types with no significant difference ($p < 0.05$) for AFP and AFP L3 but with significant difference ($p < 0.001$) for GPC3 and AAG score compared with Unifocal tumor types. In previous studies, elevated AFP-L3 has been reported to be correlated to a shorter doubling time of tumor volume, increased hepatic arterial supply, and pathologic features such as infiltrative tumor growth pattern, capsule infiltration, vascular invasion, and intrahepatic metastasis (Kumada et al., 1999 and Tada et al., 2005). AFP is not elevated in all patients with HCC. Factors such as age, sex, infection with HBV and HCV, cirrhosis and acute liver necrosis specially size and form of tumor pathology can influence AFP level. The test had a sensitivity of 39–65%, and a specificity of 76–94% in the presence of HCC in previously published studies (Daniele et al., 2004).

AFP-L3, as compared with AFP, has better sensitivity and specificity for the early diagnosis of HCC. Leerapun A et al. (2007) have demonstrated that the diagnostic specificity of AFP-L3 for early diagnosis of HCC reaches 100% when HCC patients have a cut-off value of AFP-L3 35% and serum AFP 10-200 ng/ml. In the early diagnosis of HCC at stage I or when the tumor size was <2 cm, AFP-L3 that was measured by using the μTAS method, was showed with high sensitivity (42.5 and 46.0%, respectively). Furthermore, Kobayashi et al. (2011) have indicated that when the cut-off value of AFP-L3 is 5%, sensitivity for HCC reaches 47.2% compared with AFP that is 38%. In general, it can be concluded from the research results that AFP-L3 seems to be a useful marker for early diagnosis of HCC compared with AFP alone. In the present study Serum GPC3 was more sensitive and specific than AFP and AFP L3.

Our results indicated that serum GPC3 more accurate than AFP and AFP-L3 for the diagnosis of HCC. In a meta-analysis, the pooled sensitivity and specificity of serum GPC3 for the diagnosis of HCC overall were 55.2% (52.9%-57.4%) and 84.2% (82.2-86.0%), respectively (Jia et al., 2014). For comparison, the pooled sensitivity and specificity of AFP for the same study were 34.7% (26.2%-44.1%) and 87.6% (82.6%-91.6%), respectively (Jia et al., 2014). In some other studies, the sensitivity and specificity of GPC3 in the diagnosis of HCC was found to be 77% and 96%, respectively (Libbrecht et al., 2006). The diagnostic sensitivity and specificity of AFP-L3 were 41.5% and 85.1%, respectively and many studies have investigated the role of AFP-L3/AFP, alone or in combination with AFP as a screening marker for HCC. The sensitivity of AFP-L3/AFP has been shown to vary with tumor size (Huang et al., 2013). Sun et al, (2008) when taking AFP-L3 > or = 10% as diagnostic criteria, they found the sensitivity of AFP-L3 in HCC diagnosis was 84.8% (67/79) and the specificity was 92.5% (49/53), with a total conformity rate of 87.9% compared to the confirmed clinical diagnosis. As GPC3 is detected in HCC cells but not in benign liver tissues, it has potential as a biomarker for the diagnosis of early stage HCC (Libbrecht et al., 2006 and Shafizadeh et al., 2008). Importantly, GPC3 expression appears to be independent of tumor size, as GPC3 exhibited a sensitivity of 56% in patients with early stage tumors that are < 3 cm in size (Tangkijvanich et al., 2010). Some clinical researches have indicated that the high percentage of AFP-L3 is closely related to poor differentiation and biologically malignant characteristics (especially portal vein invasion) of HCC (Khien et al., 2001 and Oka et al., 2001), and HCC patients with positive AFP-L3 would have worse liver function, poorer tumor histology, and larger tumor mass (Yamashiki et al., 1999).

Currently, no “universal” tumor marker that can detect any particular type of cancer; however, this result fails to provide a sufficient basis for cancer diagnosis. Therefore, measurements of tumor markers are usually combined (Tsai et al., 2007). In that approach in the present study the combination of serum AFP, AFP-L3 and Glypican-3 was yield AAG score. Our results indicated that, combination of GPC3, AFP and AFP-L3 in AAG score become more accurate than any studied marker alone. These results agreed with Xu et al., (2013) when reported, in ten studies the pooled sensitivity for AFP and GPC3 is 51.9% and 59.2%, respectively, while the pooled specificity for AFP and GPC3 is 94% and 84.8%, respectively. In addition, Jia et al., (2014) in nineteen studies were pooled sensitivity and specificity of serum GPC3 for the diagnosis of HCC were 55.2% and 84.2%, respectively. When combining GPC3 with AFP, pooled sensitivity and specificity were 75.7% and 83.3%, respectively. For diagnosis of early HCC, pooled sensitivity and specificity of serum GPC3 were 55.1% and 97.0%, respectively. This meta-analysis indicates that serum GPC3 has a comparable accuracy to AFP for the diagnosis of HCC, and there is an elevation in the sensitivity of diagnosis when GPC3 was combined with AFP.

CONCLUSION

The simultaneous determination of GPC3 and AFP and AFP L3 may significantly increase the sensitivity for diagnosis of HCC. Because HCC is a complex disease with multiple underlying pathogenic mechanisms caused by a variety of risk factors, it is difficult to characterize HCC with a single biomarker. Thus, signatures of a combination of GPC3 and AFP and AFP L3 may be more valuable for the diagnosis, staging, and prognosis of HCC.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

Authors would like to thank chemistry department of Faculty of Science, Menofia University, Specialized medical hospital and Tropical Medicine Department, Faculty of Medicine, Mansoura University for supporting any facilities to conduct this research.

AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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