Diagnostic accuracy of combined Glypican-3 and Alpha-Fetoprotein in regenerated, dysplastic and hepatocellular carcinoma nodules

Ayman Z. Elsamanoudy[1,5], Hatem El-Alfy[2], Salwa M. Abo El-khair[1], Asem Shalaby [3], Mahmoud A. Attia[4*], Ashraf A. El-Bindary[4]

[1]Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

[2]Tropical Medicine Department, Mansoura Faculty of Medicine, Mansoura University Hospital, Mansoura 35516, Egypt

[3]Department of Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

[4]Department of Chemistry, Faculty of Science, Damietta University, Damietta 34517, Egypt.

[5]Department of Clinical Biochemistry, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia

*Corresponding author: E-mail: mahmoudattia1472@gmail.com (M.A. Attia).

Abstract:

Hepatocellular carcinoma (HCC) is lethal common malignancy worldwide. Diagnostic procedures for HCC are serum tumour markers, different imaging techniques and histopathological examination. The present work evaluates Glypican-3 as a useful diagnostic biomarker for HCC. HCC rat model was carried out. Histopathological, biochemical and molecular evaluation of AFP and Glypican-3 was performed using immunohistochemical, ELISA and RT-PCR techniques. Serum GPC3 and AFP had significant elevation in HCC compared to degenerating, precancerous and control groups. On one hand, the sensitivity, specificity and accuracy of the control versus either degenerating or precancerous groups for serum GPC3 are higher than those for AFP are. On the other hand, the control versus HCC group has the same sensitivity, specificity and accuracy for both markers. Using RT-PCR, GPC3 and AFP were elevated in cancerous and precancerous than in degenerating group. In regression analysis, combination of AFP and GPC3 has very high NPV (100%) to differentiate either degenerated nodules or dysplastic nodules from HCC. The bottom line is that HCC diagnostic accuracy is increasing by using GPC3, which is an acceptable biomarker (at serum and molecular levels). The sensitivity for diagnosis of HCC is escalated by the simultaneous determination of GPC3 and AFP.

Keywords: Alpha-fetoprotein, Hepatocellular carcinoma, Glypican 3, Noninvasive biomarkers.

1 Introduction and Aim of the Work

A major cause for morbidity and mortality is hepatocellular carcinoma (HCC). It ranks seven in the terms of cancer types & the 3rd leading cause of cancer-related deaths. Most cases are detected at late stage by the current strategy. Thus, five-year survival rate is 10%-15% [1].

The commonest used management choice for the HCC cases is surgical resection [2]. If HCC early detected and diagnosed, it is of great clinical benefit. Well-defined non-viral and viral etiological factors are associated with HCC. HCC is linked to chronic viral infection either hepatitis B virus (HBV) or hepatitis C virus (HCV), chemical carcinogenic substances (e.g. aflatoxins), and various environmental factors. In addition, host factors, that cause liver injury, are linked etiologically to HCC [3].

On top of chronic hepatitis or cirrhosis, with existence of hepatocytes' regeneration and continuous inflammation, HCC is developed due to chromosomal aberrations and/or the non-random accumulated genetic alterations. This multi-steps tumorigenic process progresses from hyperplasic change, to dysplasia then early HCC. Finally, this process results in full-developed HCC [4], [5].

The present strategy does not work in terms of detecting the early disease. Serum alpha-fetoprotein (AFP) estimation and 6 monthly abdominal ultrasound scan (USS) represent this strategy. This strategy is inactive because abdominal USS is user dependent and technically difficult in cirrhotic nodular livers. Moreover, the diagnostic power of AFP is insufficient as it is only elevated in 40-60% of HCC patients [6].

Glypican-3 (GPC3) is a new alternative liver cancer biomarker. Glypicans are heparan sulfate proteoglycans family, which are linked by a glycosyl-phosphatidylinositol anchor to the exocytoplasmic surface of the plasma membrane. In mammals, there are six identified glypicans (GPC1-GPC6). Researches reported that GPC3 is down expressed in ovarian cancinoma, cancer breast and lung adenocarcinoma while in HCC, it is up regulated [7].

GPC3 have been detected in nearly 50% of HCC cases and 33% of HCC sero-negative AFP patients, but it does not exist in hepatocytes of healthy subjects nor in patients with non-malignant hepatopathy [8].

As an oncofetal antigen, GPC3 is considered as a valuable biomarker for HCC detection, especially, in small or poorly differentiated HCC. There are ambiguous factors related to the dynamic changes of GPC3 and its mRNA expression at HCC early stages [1], [9].

Consequently, this work evaluates GPC3 as a tumour biomarker for HCC and the possible use of this marker for early diagnosis of HCC. In addition, this paper compares the sensitivity and specificity of GPC3 to the traditionally used marker AFP with novel use of their combined levels to differentiate between different hepatic nodules.

2 Materials and Methods:

2.1 Animals:

The method of Yao et al., [10] describes the hepatoma model used in this work and Yao et al., [1] confirms it. Theodor Bilharz Research Institute–animal house, Giza, Egypt, sold 48 male Sprague-Dawley rats in favour of this paper. These rats weighed 120-200 g. They were divided randomly into control group (12 rats) and experimental group (36 rats). All animals were reserved in cages with temperature-controlled environment and a 12-h light-dark cycle and had free access to food and water throughout the study. The control rats were fed with standard grains, while the experimental group rats were additionally given 2-fluorenylacetamide (2-FAA - 0.05%, Sigma, USA). The clinical monitoring of rats focuses on survival, weight loss and recording their clinical signs. Every two weeks, 4 rats of the experimental group and 2 control rats were sacrificed in order to monitor tumor development. The investigation of GPC3 and AFP, by ELISA technique, from the collected blood samples was performed.

For histological examination, part of the liver tissues were fixed with 10% formalin then stained with hematoxylin and eosin (H & E). A liquid nitrogen frizzed the rest of liver tissues and they stored at -80°C for further RNA extraction and RT-PCR. The guidelines for animal care approved by Ethical Committee of Mansoura University, Egypt; are the standards of all animal experimental care used in this study.

2.2 Methods:

2.1.1 Pathological Classification using Haematoxylin and Eosin (H & E):

All rat liver tissues were stained with H & E stain for pathological assessment. After fixation with 10% formalin, tissue specimens were embedding in paraffin, 4 µm sectioned, then deparaffinized by xylene & dehydrated using ethanol gradient solutions. Finally, stained with H & E and pathologically examined.

Serum biochemical parameters:

Rat specific ELISA kits: GPC3 ELISA kit (Elabscience Biotechnology Co., Ltd, subpackaged, China-Catalog No: E-EL-R0974) and AFP ELISA kit (Elabscience Biotechnology Co., Ltd, subpackaged, China-Catalog No: E-EL-R0153) were used for detection of serum GPC3 and AFP according to the manufacturer's instructions. The optical density (OD) was spectrophotometrically assessed at 450 ± 2 nm. Detection range for GPC3 is 0.156-10 ng/mL while for AFP is 0.313-20 ng/mL. Comparison of the samples' OD to the standard curve used for calculation of the concentration of GPC3 in the samples.

Molecular Biology-Based assay for GPC3 and AFP:

Total RNA extraction: homogenized liver tissue sample (about 25 mg) with one ml trizol reagent (MRC, USA), was chloroform mixed and centrifuged at high speed. Then, supernatant collected & isopropanol (equal volume) was

added. After washed by 70% cold ethanol, centrifugation pelleted RNA, was resuspended in RNase-free water. SmartSpec TM plus spectrophotometer (Bio-Rad, USA) measured the concentration of total RNA and A260/280 ratio.

2.2.2 Synthesis of cDNA:

2 µg of extracted total RNA were used for cDNA synthesis using reverse transcriptase (Gibco BRL, USA). Synthesized cDNA was stored at -20 ℃ for further GPC3 and AFP gene expression analysis.

2.2.3 Detection of GPC3 gene expression (nested PCR):

GPC3 GenBank sequence (NM_012774) was used for designing GPC3 primers. The external primers sequences were: GPC3 forward: 5'-GTGTGGTAGAGATCGACAAG-3' and GPC3-reverse: 5'-AGCGCAGTTGGTTCTTCACT-3'. The internal primers sequences were: GPC3-forward: 5'-TATGTGCAGAAGAACGGAGG-3' and GPC3 reverse: 5'-CTCAGGGCCCTT CATTTTCA-3'), giving a PCR product of 372 bp. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used in all samples as a reference control gene. The internal control primers were GAPDH- forward: 5'-AAGGTCATCCCAGAGCTGAA-3' and GAPDH-reverse: 5'-GAGGGCCTCTCTCTTGCTCT-3', and the amplified fragment was 595 bp. The 1st PCR reaction was performed with synthesized cDNA as a template and the external primers. The PCR cycling was: hot start (94℃, 5 minutes), then 38 cycles: 94℃ for 10 sec., 50℃ for 30 sec., 72℃ for 1 min., and final extension (70℃, 10 min.). The 2nd step PCR reaction was performed with the template (1st PCR product) and internal primers using similar PCR protocol.

2.2.4 Detection of AFP gene expression (RT-PCR) [11]:

The expression of AFP mRNAs was analysed by semi-quantitative RT-PCR. Sequence of rat-specific primers for AFP: 5'- GCT GAA CCC AGA GTA CTG CAC-3', and 5'-GAC ACG TCG TAG ATG AAC GTG-3', and the amplified fragment was 443 bp. A PCR reaction mix (50 ul) was performed, using cDNA as a template. PCR reaction conditions were: denaturation (94°C, 4 min.), 35 cycles; denaturation (94°C, 30 sec.), annealing (68°C, 30 sec.), extension (72°C, 30 sec.), then, one cycle (72°C, 7 min).

2.2.5 Analysis of Amplified PCR Products:

Amplified PCR products were subjected to agarose gel electrophoresis (1.5%), ethidium bromide stained, subjected to UV Trans-illuminator and photographed with standard conditions. No RT and no RNA control reactions were used to exclude genomic or target gene contamination respectively. Calculating the relative ratio of expression of the specific genes was estimated in relation to the internal control gene GAPDH.

2.2.6 Immunohistochemistry of GPC3 and AFP:

An UltraSensitiveTM S-P (streptavidin-peroxidase) kits, anti-GPC3 monoclonal antibody (Abcam, UK, USCN Life Science, China), and monoclonal antibody of anti-AFP (DAKO, Glostrup, Denmark) were used. The –ve control with phosphate-buffered reagent (0.01 mol/L) instead of 1ry and 2ry antibodies, and S-P reagent. Liver GPC3 and AFP expression was semi quantitatively measured as +ve cell percentage, and grouped as: diffuse +ve staining (+++) of >50%; moderate staining (++) of 15-50%; weak staining (+) of 5-15%; and –ve staining (-) of <5% of total cells.

2.2.7 Statistical Analysis

The computer program SPSS (Statistical package for social science) version 17.0 analyzed, coded and tabulated data. Mean, standard deviation (SD) and frequency (number, percent) are the forms of the calculated descriptive statistics. The significance of difference was tested with ANOVA (analysis of variance) in terms of the statistical comparison between the different groups. The comparison aimed to relate >two groups of numerical (parametric) data followed by post-hoc tukey test for multiple comparisons. Different cutoff points were used to examine the sensitivity and specificity of GPC3 and AFP for early diagnosis of HCC. This process used Reciprocal operative (ROC) curve analysis in order to determine the diagnostic power of each test and the best cut-off point. A P value ≤ 0.05 was considered statistically significant.

3 Results:

This research was performed using 48 male Sprague-Dawley rats. The rat liver samples were histopathologically classified into 4 groups using H & E staining; control (12, 25%), Degenerating (19, 39%), Precancerous (9, 19%) and HCC (7, 17%). One rat died during the experimental course. All serum samples were tested for estimating GPC3 and AFP levels against sera collected from 12 control rats (table 1).

TABLE 1

SERUM GPC3 AND AFP LEVELS FOR DIFFERENT PATHOLOGICAL GROUPS

		Serum GPC3		Serum AFP	
		Mean	±SD	Mean	±SD
Groups	Control	15.13	3.09	11.53	2.40
	Degenerating	21.31	5.02	15.89	6.24
	Precancerous	24.82	5.77	17.54	3.73
	HCC	34.40	6.87	24.46	5.77
	ANOVA P value	<0.001		<0.001	
	P1 (Deg. Vs Control)	0.01		0.1	
	P2 (Precan. Vs Control)	0.001		0.045	
	P3 (HCC Vs Control)	<0.001		<0.001	
	P4 (Precan. Vs Deg.)	0.34		0.8	
	P5 (HCC Vs Deg.)	<0.001		0.001	
	P6 (Precan. Vs HCC)	0.002		0.03	

P= Probability, P = significance when<0.05, Test used: ANOVA followed by post-hoc tukey for multiple comparisons; P1= significance between Control & Degenerating groups, P2= significance between Control & precancerous groups P3: significance between Control & HCC group, P4 = significance between Degenerating & precancerous groups, P5 = significance between Degenerating & HCC group, P6 = significance between Precancerous & HCC group.

Serum GPC3 and AFP showed statistically significant elevated results in HCC compared to degenerating,

precancerous and control groups.
The ROC curve analysis is used to determine the best cutoff point in addition to the diagnostic power of each test during the hepatocarcinogenesis progression (table 2).

TABLE 2

DIAGNOSTIC PERFORMANCE OF SERUM GPC3 AND AFP (ROC CURVE ANALYSIS)

PPV = Positive Predictive value %, NPV = Negative Predictive value %, CI 95% = Confidence Interval.

		AUC (CI 95%)	Cutoff Value	Sensitivity	Specificity	PPV	NPV	Accuracy
Control vs Degenerating groups	GPC3	0.83 (0.69-0.97)	16.6	77.8	66.7	61.3	78.9	74.2
	AFP	0.73 (0.55-0.91)	12.8	68.4	58.3	72.2	53.8	64.5
Control vs Precancerous groups	GPC3	0.95 (0.86-1.00)	18.5	88.9	83.3	80.0	90.9	85.7
	AFP	0.88 (0.72-1.00)	13.61	77.8	75.0	70.0	81.8	76.2
Control vs HCC groups	GPC3	1.00 (1.00-1.00)	21.8	100	100	100	100	100
	AFP	1.00 (1.00-1.00)	16.48	100	100	100	100	100

Regarding to control versus degenerating group using ROC curve, we assessed the diagnostic accuracy of serum AFP and GPC3. The cut-off of serum AFP was 12.8 ng/mL yielded sensitivity 68.4%, specificity 58.3% and efficiency 64.5% while, the cut-off of serum GPC3 was 16.6 ng/mL yielded sensitivity 77.8%, specificity 66.7% and efficiency 74.2%. Regarding to control versus precancerous group using ROC curve, we assessed the diagnostic accuracy of serum AFP and GPC3. The cut-off of serum AFP was 13.61 ng/mL with sensitivity 77.8, specificity 75.0% and efficiency 76.2% while, the cut-off of serum GPC3 was 18.5 ng/mL with sensitivity 88.9%, specificity 83.3% and efficiency 85.7%.
Regarding to control versus HCC group using ROC curve, the cut-off of serum AFP was 16.48 ng/mL and for serum GPC3 was 21.8 ng/mL with sensitivity 100%, specificity 100% and efficiency 100%. The sensitivity, specificity and accuracy of the control vs degenerating or precancerous groups for serum GPC3 are higher than those for AFP; while the control vs HCC groups have the same sensitivity, specificity and accuracy for both markers (Table 2).

The results of GPC3 using RT-PCR as well as AFP were found to be higher in the cancerous and precancerous than in the degenerating groups. While GPC3 expression was significantly increase in degenerative group than control, AFP expression was not significantly different (Table 3), indicating that GPC3 but not AFP expression begins to increase and differs with early degenerative changes during hepatic carcinogenesis.

TABLE 3

GPC3 AND AFP GENE EXPRESSION (RT-PCR) IN DIFFERENT PATHOLOGICAL GROUPS

P = Probability, P = significance when<0.05, Test used: ANOVA followed by post-hoc tukey for multiple comparisons; P1= significance between Control & Degenerating groups, P2= significance between Control & precancerous groups

		GPC3		AFP	
		Mean	±SD	Mean	±SD
Groups	Control	0.47	0.31	0.29	0.28
	Degenerating	0.65	0.28	0.38	0.15
	Precancerous	0.77	0.53	0.45	0.190
	HCC	0.93	0.37	0.57	0.47
	ANOVA P value	<0.001		<0.001	
	P1 (Control Vs Deg.)	<0.001		0.13	
	P2 (Control Vs Precan)	<0.001		0.002	
	P3 (Control Vs HCC)	<0.001		<0.001	
	P4 (Deg. Vs Precan.)	0.01		0.08	
	P5 (Deg. Vs HCC)	<0.001		<0.001	
	P6 (Precan. Vs HCC)	0.001		0.016	

P3: significance between Control & HCC group, P4 = significance between Degenerating & precancerous groups, P5 = significance between Degenerating & HCC group, P6 = significance between Precancerous & HCC group.

The hepatic sections of rats stained for GPC3 and AFP are corresponding to the histopathological changes and HCC development in the liver for each group are shown in figure (1 and 2). The positive liver GPC3 protein gave brown staining, distributed mainly in cytosol and membrane. The closer to cancerous tissue, the stronger the expression of GPC3 was. After immunostaining quantification, the incidence of liver GPC3 and AFP expression and their intensity during malignant transformation are listed in table 4. Both markers expression are differ significantly with different hepatic carcinogenesis. However, AFP expression does not differ significantly from degenerative to precancerous stage.

3.1 BUILD UP A MODEL FOR USING COMBINED GLYPICAN-3 AND AFP FOR DIFFERENTIATING BETWEEN REGENERATIVE AND /OR DYSPLASTIC NODULES VERSUS CANCEROUS NODULES:

Step 1 from regenerative nodule to HCC nodule, regression demonstrate the following equation for combined AFP and GPC3 (GPC3 serum level * 8.336 + AFP serum level *4.775 + -1.938E2 =); while in step 2 from dysplastic to HCC nodules, regression reveals the following equation for combined AFP and GPC3 (GPC3 serum level * 0.554 + AFP serum level *0.114 + - 11.572 =).

TABLE 4

LIVER GPC3 AND AFP EXPRESSION INTENSITY (IMMUNOHISTOCHEMICAL) AT DIFFERENT STAGES OF HEPATOCYTE MALIGNANT TRANSFORMATION

		GPC3		AFP	
		Mean	±SD	Mean	±SD
Groups	Control	0.249	0.006	0.22	0.006
	Degenerating	11.45	2.56	6.29	1.30
	Precancerous	15.11	3.75	8.64	3.31
	HCC	20.68	2.24	13.45	4.04
	ANOVA P value	<0.001		<0.001	
	P1 (Deg. Vs Control)	<0.001		<0.001	
	P2 (Precan. Vs Control)	<0.001		<0.001	
	P3 (HCC Vs Control)	<0.001		<0.001	
	P4 (Precan. Vs Deg.)	0.003		0.07	
	P5 (HCC Vs Deg.)	<0.001		<0.001	
	P6 (Precan. Vs HCC)	<0.001		0.001	

P = Probability, P = significance when<0.05, Test used: ANOVA followed by post-hoc tukey for multiple comparisons; P1= significance between Control & Degenerating groups, P2= significance between Control & precancerous groups P3: significance between Control & HCC group, P4 = significance between Degenerating & precancerous groups, P5 = significance between Degenerating & HCC group, P6 = significance between Precancerous & HCC group.

3.2 DIFFERENTIATION BETWEEN REGENERATIVE NODULES VERSUS HCC NODULES:

Our study reveals that either GPC3 or combinations (AFP with GPC3 in regression model) have very high NPV and PPV reach 100% with cut-off point 17.46 and 0.607 respectively (table 5).

TABLE 5

REGRESSION ANALYSIS FOR COMBINED USE OF BOTH AFP AND GPC3 IN DEGENERATED AND DYSPLASTIC NODULES VERSUS HCC NODULES

Group	Cut off point	Sensitivity	Specificity	Accuracy	PPV	NPV	False Negative	False Positive
From nodule to HCC								
GPC3	17.46	100%	100%	100%	100%	100%	Zero	Zero
AFP	10.37	100%	87.6%	96%	95%	100%	Zero	5%
Combination	0.607	100%	100%	100%	100%	100%	Zero	Zero
From pre-cancer to HCC								
GPC3	17.54	75%	100%	86.6%	100%	77.7%	22.3%	Zero
AFP	9.6	75%	85.7%	80%	85.7%	75%	25%	14.3%
Combination	0.54	100%	87.5%	93.3%	87.5%	100%	Zero	12.5%

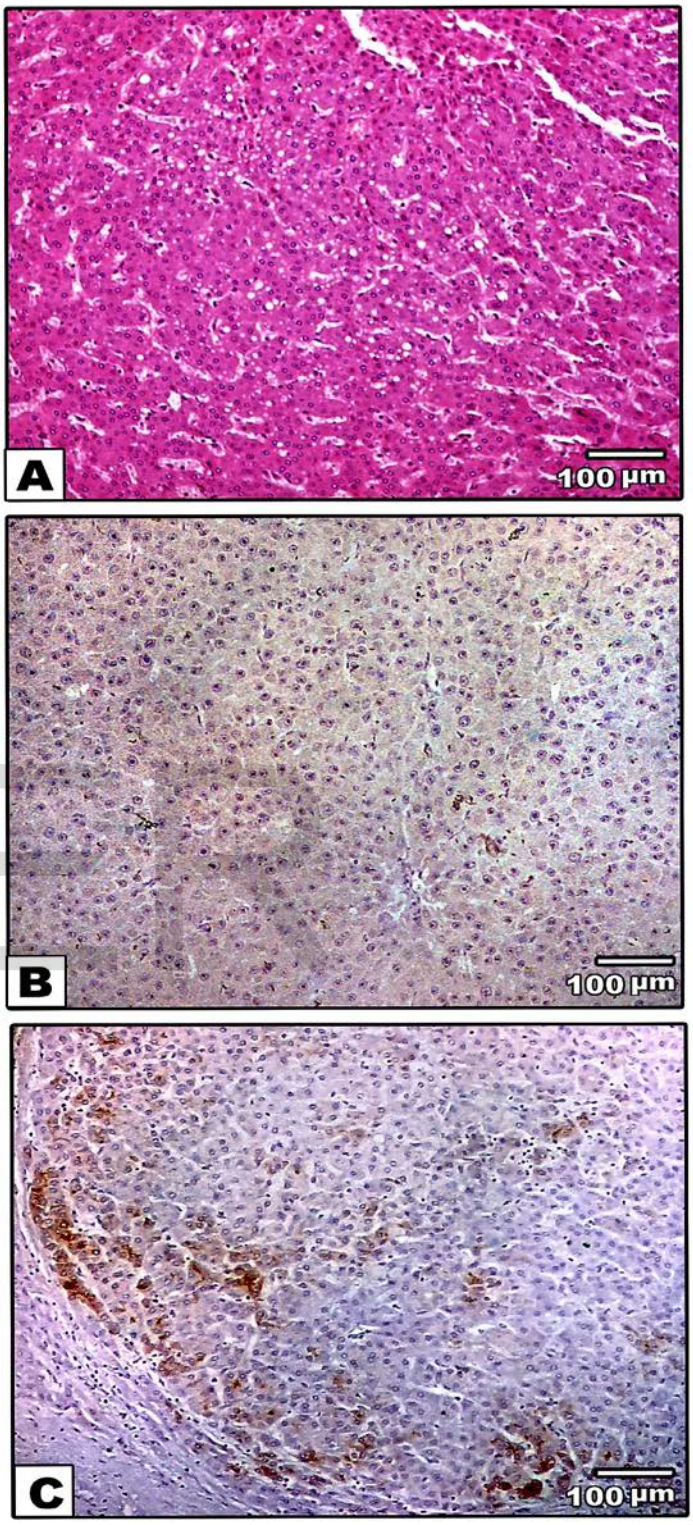

Fig. 1. Precancerous stage

(A) Precancerous liver stage section stained with H&E. (B) AFP immunostaining of the precancerous liver section showing negative reaction (DAB 100x). (C) GPC3 immunostaining of the precancerous liver section showing medium positive intensity reaction (DAB 100x).

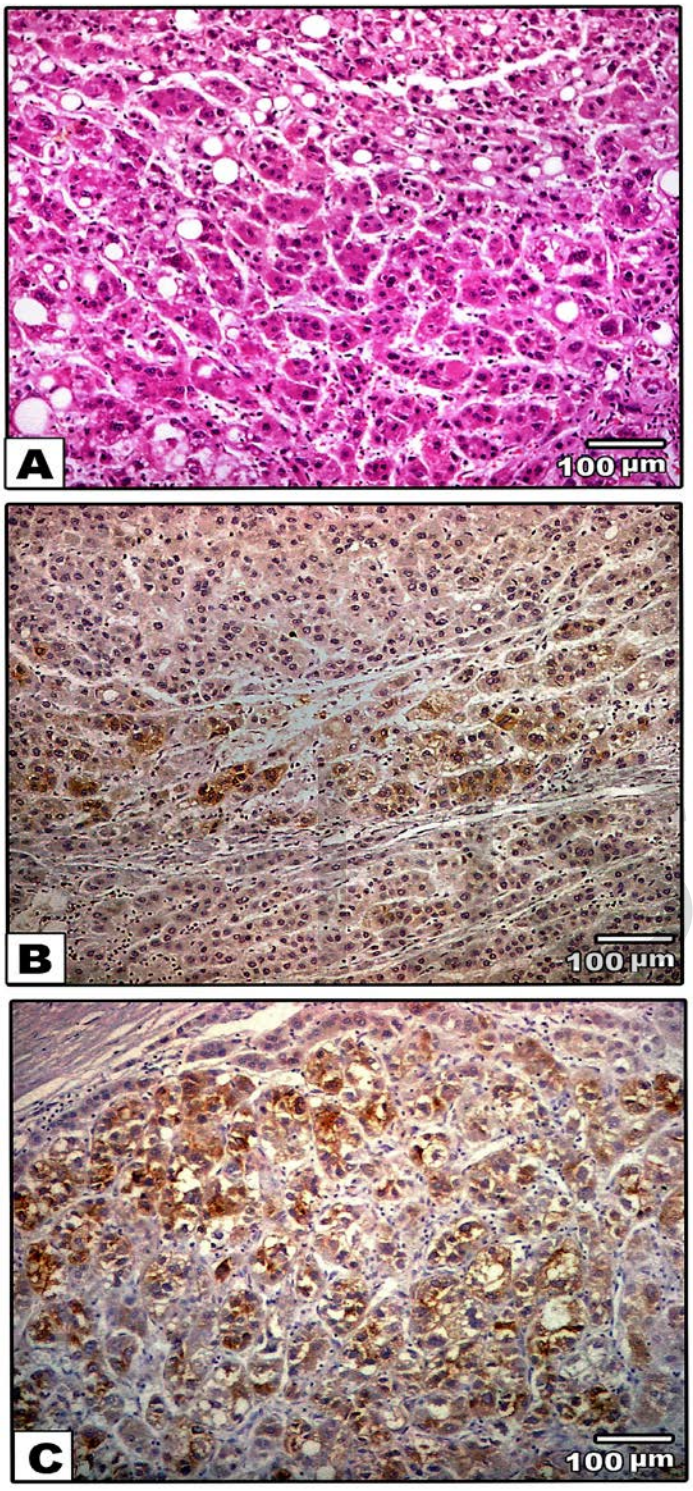

Fig. 2. Hepatocellular carcinoma (HCC) stage

(A) HCC stage section stained with H&E. (B) AFP immunostaining of the HCC section showing positive reaction (DAB 100x). (C) GPC3 immunostaining of the HCC section showing high positive intensity reaction (DAB 100x).

3.3 Differentiation between dysplastic nodules versus HCC nodules:

This study demonstrate that combinations (AFP with GPC3 in regression model) have very high NPV reach 100% (false negative zero) with cut-off point 0.54, which is more accurate than use each biomarker alone. AFP and GPC3 has false negative percent 25% and 22.3% respectively (table 5).

4 Discussion

Globally, the most common primary liver cancer is HCC. In Egypt, it is the seventh cancer among females and the second most cancer site among males after bladder cancer [13]. These statistics resulted from the high prevalence of HCV among the Egyptian population [5]. When curative therapies are available, HCC is asymptomatic & at early stages of the disease. The effect of therapy, patient survival and quality of life improved significantly by accurate detection and diagnosis of early HCC [14]. Therefore, HCC early diagnosis and reduced misdiagnosis depend on & improved by screening molecular biomarkers with high specificity and sensitivity [15].

Presently, diagnostic imaging techniques and estimation of serum biomarkers are the methods for detecting HCC clinically. For screening, ultrasonography is widely used because of its diagnostic accuracy, non-invasiveness, patients' acceptance and its average cost. However, this technique depends mainly on the user's experience [16]. In up to 90% of cases, HCC has a cirrhotic background. Presence of fibrotic septa and regenerative nodules might hinder small tumors' identification by ultrasonography [17]. Moreover, this technique displays limitations as regards sensitivity and specificity, especially at the early stage of the disease [14].

Tumor biomarkers estimation is important for HCC management. In general, a clinically valuable biomarker achieves sensitivity and specificity level ≥ 90%, should be non-invasive and cost-effective. Therefore, the desired biomarker should be tumor-specific and detected easily in body fluids (serum, plasma, bile) [18]. Des-gamma-carboxy-prothrombin (DCP), Lens culinaris agglutinin A-reactive fraction of alpha-fetoprotein (AFP-L3) and AFP represent HCC-specific tumor markers [19]. DCP level, at a cutoff value of 40 mAU/ml, has a moderate sensitivity (61.5%) but high specificity (94.7%) for HCC detection in high-risk people [20].

Serum AFP is used widely in HCC screening programs and was presented in international HCC surveillance guidelines. However, AFP has major limitation: it is not significantly increased in about 50% of HCC patients, low diagnostic accuracy, sensitivities range 18–60% and specificity of ~85–90%. Therefore, HCC surveillance guidelines recently excluded AFP [14]. GPC3 is expressed abundantly in placental and fetal tissues (lung, liver, kidney). However, its expression is reduced significantly in adult tissues [21]. As glypicans interact with and modulate growth factors' activities; they play vital role in cell growth, differentiation and migration [22]. As an oncofetal antigen, GPC3 is proposed to be a useful biomarker for HCC

detection, especially the poorly-differentiated or small HCC [23].

To achieve our previously mentioned aim (to evaluate GPC3 as a useful biomarker for HCC diagnosis in comparison to routinely used AFP), a rat hepatoma model was successfully induced and pathologically confirmed. The 48 Sprague-Dawley rats enrolled in this research were histopathologically classified into 4 groups: control, degenerating, precancerous and HCC group.

In the current study, the target GPC3 was resolved using a specific monoclonal antibody for circulating GPC3 identification in serum samples of different studied groups. The heterodimer mature GPC3 was expressed as a GPI-anchored protein closely to the cell membrane & has two HS chains linked to the C-terminal region [24]. GPC3 might be released into the extracellular environment as a glycated form with a molecular weight >100 kDa or as a 50 kDa protein fragment without HS chain [25]. In the rat hepatoma model in our study, the brown staining of GPC3 was primarily distributed in the cytosol parallel with the staining of AFP. As close to the tumor, the brown staining was stronger, indicating its potential function as a molecular chaperon. In addition, GPC3 staining was present in the endoplasmic reticulum & Golgi around the nucleus. This result highlights that GPC3 is over-expressed in HCC. This was recorded in other similar researches, and refers to GPC3 as a promoting factor to HCC [1], [26].

HCC develops from normally GPC3 expressing tissues during their fetal stage, and therefore, renewed GPC3 expression occurs with malignant transformation [8]. By GPC3 immunolabelling, Capurro et al., [27] recorded that GPC3 was positive in 72% of HCCs and undetectable in normal hepatic tissue, cirrhotic tissue or benign hepatic lesions. Other researchers evaluated GPC3 got similar results [28], [29]. Moreover, our work revealed that GPC3 was over-expressed in early HCC and therefore, could be a sensitive and specific marker for early stage HCC detection.

GPC3 is expressed only in tumor cells & not in normal hepatic cells, so, it might be a potential target for HCC therapy. Giordano and Columbano [30], tried injection of monoclonal GPC3 antibodies in late HCC stage with various GPC3 levels, and they recorded that cancer progression time in elevated GPC3 was more than that in those with reduced GPC3 expression. Despite of being a cell-surface biomarker, GPC3 might be secreted into the serum by breaking its GPI anchor with the lipase Notum [31]. Therefore, GPC3 represents a valuable diagnostic biomarker [32], [33].

In the present study, the expression of liver GPC3 was significantly elevated in degenerative, precancerous and HCC groups than in control group; referring to GPC3 involvement in hepatic carcinogenesis. Although the mechanism is not yet obvious, GPC3 over-expression might be a promising molecular biomarker for early detection of HCC. These results agreed with Qiao et al., [32] who analyzed serum levels of three biomarkers: GPC3, Human-Cervical-Cancer-Oncogene (HCCR) and AFP, for HCC diagnosis in 189 samples (101 HCC patients, 40 cirrhotic cases, 18 hepatitis patients and 30 healthy control subjects). They reported GPC3 as the best among them.

Chen et al., [33] estimated serum GPC3 in 1037 subjects (155 HCC cases, 180 chronic hepatitis, 124 liver cirrhotic patients, 442 non-HCC cancers & 136 healthy subjects). The mean GPC3 level was: in HCC, 99.94 ± 267.2 ng/ml; in chronic viral hepatitis, 10.45 ± 46.02 ng/ml; liver cirrhotic patients, 19.44 ± 50.88 ng/ml; non-HCC cancers, 20.50 ± 98.33 ng/ml & in healthy subjects, 4.14 ± 31.65 ng/ml. GPC3 acts as a co-receptor for specific ligands, e.g. Wnt and FGF, and therefore, stimulate some signaling pathways having role in HCC development and invasion. Many studies reported that GPC3 might promote hepatoma cells' growth [34]. Capurro et al., [35] proposed that GPC3 promotes hepatoma cells' growth through activation of Wnt/β-catenin signaling pathway.

Li et al., [34] proved that in HCC cells, ectopic GPC3 might elevate c-Myc expression, one typical target for the Wnt/β-catenin signaling pathway; and c-Myc could directly stimulate transcription of GPC3. Zittermann et al., [36] proved that soluble GPC3 inhibited the in vivo HCC cells growth via Wnt signaling pathway blocking. In other words, they proposed that this activity might be presented only with GPC3 attachment to cell membrane.

In addition, GPC3 might stimulate H suppression CC cells' growth by promoting other signaling pathways. Sun et al., [37] proved that GPC3 suppression leads to cell proliferation inhibition and enhancement of apoptosis via up-regulation of TGF-β2. As GPC3 is detected only in HCC cells, and not in benign liver conditions, it could be used as a potential biomarker for early HCC screening and diagnosis [38].

Several studies reported that GPC3 expression (at mRNA & protein levels) is elevated in a high percentage of patients of HCC [8], [26], [27], [39], [40]. Although GPC3 mRNA is broadly expressed, it still might serve as a potential tissue tumor biomarker for HCC.

In the present study, the incidence of up-regulated GPC3 mRNA dynamically elevated as hepatocytes transformed from normal to degenerative to precancerous & finally, cancer lesion. The stimulation of GPC3 mRNA expression was evident in hepatic tissue samples (cancerous and precancerous lesions, in areas of the degenerative samples) but not in normal samples, referring to GPC3 mRNA over-expression as a sensitive and specific HCC marker that could differentiate it from other benign or cancerous liver lesions. Yan et al., [41] reported +ve AFP mRNA expression in HCC, hepatitis B, and cirrhotic patients as 56, 5, and 10%, respectively. However, it was not reported in control subjects, hepatic hemangiomas, or metastatic hepatic samples. Therefore, GPC3 expression is considered HCC-specific.

The dynamic expression changes of GPC3 during HCC development were co-related to the liver histopathology, indicating that high GPC3 expression is an early molecular change co-existed with hepatocytes malignant transformation. Therefore, GPC3 gene expression analysis might be useful for early HCC diagnosis. In the present

work, the sensitivity, specificity and accuracy of the control versus degenerating and the control versus precancerous groups for serum GPC3 using ELISA are higher than those for AFP; while the control versus HCC groups have the same sensitivity, specificity and accuracy for both markers.
Our results agreed with Qiao et al., [32]. With 26.8 ng/mL as the cut-off level for HCC detection (nearly similar to our cut off level 21.48 ng/ml), GPC-3 had a sensitivity of 51.5% and a specificity of 92.8% but the sensitivity and specificity of our study are higher. Moreover, Xu et al., [42] in their meta-analysis including ten studies, reported that a pooled sensitivity for AFP and GPC3 was 51.9% and 59.2%, respectively, while the pooled specificity for AFP and GPC3 was 94% and 84.8%, respectively.

In addition, Jia et al., [43] conducted a meta-analysis including nineteen studies. The sensitivity and specificity of serum GPC3 for HCC diagnosis was 55.2% and 84.2%, respectively. When combining GPC3 with AFP, pooled sensitivity and specificity were 75.7% and 83.3%, respectively. This meta-analysis proved that serum GPC3 has a comparable accuracy as AFP with an increase in its sensitivity when both markers are combined. Our study verified the use of combined GP-3 and AFP in regression model for differentiating between different hepatic nodules, this combined model reach high accuracy for dysplastic versus HCC nodules that solve many clinical problems in this issue.
In this regards the combined model can help in early diagnosis of HCC, which will be reflected on clinical decision of nodules type have been seen in ultrasound, thus early treatment can be initiated and improve patients survival .

5 CONCLUSION:

The results of our study for serum GPC3 as well as its expression at the level of mRNA using RT-PCR, which was additionally localized by examination of GPC3 in rat liver tissues using immunohistochemistry; reveals that: GPC3 could be used as a conventional serum and molecular biomarker for the diagnosis of HCC, thus could elevate the accuracy of diagnosis. The co-determination of GPC3 and AFP might significantly elevate the sensitivity for early detection of HCC and differentiate it from dysplastic nodules; which reach zero for false negative and accuracy of 93.3%.

6 AUTHOR DISCLOSURE STATEMENT

The authors in this work declared that they have nothing to disclose regarding funding or conflicts of interest with respect to this article.

7 REFERENCES

1- Yao M., Yao D., Bian Y., Zhang C., Qiu L., Wei W. and Sai W. Oncofetal antigen glypican-3 as a promising early diagnostic marker for hepatocellular carcinoma. Hepatobiliary Pancreat. Dis. Int. 10: 289-294, 2011.

2- Poon D., Anderson B.O., Chen L.T., Tanaka K., Lau W.Y., Van C.E., Singh H. and Chow W.C. Management of hepatocellular carcinoma in Asia: consensus statement from the Asian Oncology Summit 2009. Lancet Oncol. 10 (11): 1111-1118, 2009.

3- Nanis S.H., Dina S.A., Hayam A.A. and Dina M.S. Hepatocellular carcinoma in Egypt: Epidemiological and histological properties. Menoufia Medical Journal. (28): 718-724, 2015.

4- Kim J.U., Shariff M.I., Mary M.E., Maria Gomez-Romero, Elaine H., Jane I.C., Haddy K.S., Ramou N. and Simon D.T. Hepatocellular carcinoma: Review of disease and tumor biomarkers. World J. Hepatol. 8 (10): 471-484, 2016.

5- Abd El-Rahman A.E., Abdel-Aziz H.R. and Hegazy A.A. Immunohistochemical Distinction of Hepatocellular Carcinoma Using Arginase-1, Hepatocyte Paraffin Antigen -1 and Glypican-3. Journal of Tumor. 4 (1): 359-366, 2016.

6- Bird T.G., Dimitropoulou P., Turner R.M., Jenks S.J., Cusack P., Hey S., Blunsum A., Kelly S., Sturgeon C., Hayes P.C. and Bird S.M. Alpha Fetoprotein Detection of Hepatocellular Carcinoma Leads to a Standardized Analysis of Dynamic AFP to Improve Screening Based Detection. PLoS One. 11(6): e0156801, 2016.

7- Sung Y.K., Hwang S.Y. and Park M.K. (2003): Glypican-3 is over expressed in human hepatocellular carcinoma. Cancer Sci. 94: 259-262, 2003.

8- Nakatsura T., Yoshitake Y., Senju S., Monji M., Komori H., Motomura Y., Hosaka S., Beppu T., Ishiko T., Kamohara H., Ashihara H., Katagiri T. and Furukawa Y. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun. 306: 16-25, 2003.

9- Yukihiro H. and Hiroaki K. Glypican-3 is a prognostic factor and an immunotherapeutic target in hepatocellular carcinoma. World J. Gastroenterol. 22 (1): 275-283, 2016.

10- Yao D., Dong Z.Z., Wu X.H., Wu W., Qiu L.W., Wang H.M. and Meng X.Y. Abnormal expression of hepatoma-derived γ-glutamyltransferase subtyping and its early alteration for carcinogenesis of hepatocytes. Hepatobiliary Pancreat. Dis. Int. 3 (4): 564-570, 2004.

11- Meier V., Tron K., Batusic D., Elmaouhoub A. and Ramadori G. Expression of AFP and Rev-Erb A/Rev-Erb B and N-CoR in fetal rat liver, liver injury and liver regeneration. Comparative Hepatology. 5 (2):1-12, 2006.

12- Ziada D.H., El Sadany S., Soliman H., Abd-Elsalam S., Salama M., Hawash N., Selim A., Hamisa M. and Elsabagh H.M. Prevalence of hepatocellular carcinoma in chronic hepatitis C patients in Mid Delta, Egypt: A singlecenter study. J Egypt Natl Canc Inst. 28(4):257-262, 2016.

13- Anwar W.A., Khaled H.M., Amira H.A., El-Nezami H. and Loffredo C.A. Changing pattern of hepatocellular carcinoma (HCC) and its risk factors in Egypt: Possibilities for prevention. Mutation Research. 659: 176-184, 2008.

14- Reichl P. and Mikulits W. Accuracy of novel diagnostic biomarkers for hepatocellular carcinoma: An update for clinicians. Oncol Rep. 36 (2):613-625, 2016.

15- Dong Z., Yao M., Wang L., Yang J. and Yao D. Down-regulating glypican-3 expression: Molecular-targeted therapy for hepatocellular carcinoma. Mini Rev Med Chem. 14:1183-1193, 2014.

16- McKillop I.H. and Schrum L.W. Role of alcohol in liver carcinogenesis. Semin Liver Dis. 29 (2):222-232, 2009.

17- Gustavo F., Patricia A. , Manuel R., José L. and Manuel M. Biomarkers for hepatocellular carcinoma: diagnostic and therapeutic utility. Hepat. Med. 7: 1-10, 2015.

18- Tsuchiya N., Sawada Y., Endo I., Saito K., Uemura Y. and Nakatsura T. Biomarkers for the early diagnosis of HCC. WJ Gastroenterol. 21 (37):10573-10583, 2015.

19- Toyoda H., Kumada T., Tada T., Sone Y., Kaneoka Y. and Maeda A. Tumor Markers for Hepatocellular Carcinoma: Simple and Significant Predictors of Outcome in Patients with HCC. Liver Cancer. 4:126-136, 2015.

20- Huang T.S., Shyu Y.C., Turner R., Chen H.Y. and Chen P.J. Diagnostic performance of alpha-fetoprotein, lens culinaris agglutinin-reactive alpha-fetoprotein, des-gamma carboxyprothrombin, and glypican-3 for the detection of hepatocellular carcinoma: a systematic review and meta-analysis protocol. Syst Rev. 6; 2:37, 2013.

21- Filmus J. and Capurro M. Glypican-3: a marker and a therapeutic target in hepatocellular carcinoma. FEBS J. 280:2471-2476, 2013.

22- Kandil D. and Cooper K. Glypican-3: A novel diagnostic marker for hepatocellular carcinoma and more. Adv. Anat. Pathol. 16: 125-129, 2009.

23- Iglesias V., Centeno G. and Pascucceli H. Expression pattern of glypican-3 (GPC-3) during human embryonic and fetal development. Histol. Histopathol. 23: 1333-1340, 2008.

24- Filmus J., Capurro M. and Rast J. Glypicans. Genome Biol. 9:224, 2008.

25- Hippo Y., Watanabe K., Watanabe A., Midorikawa Y., Yamamoto S., Ihara S., Tokita S., Iwanari H., Ito Y. and Nakano K. Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res. 64:2418-2423, 2004.

26- Zhu Z.W., Friess H., Wang L., Abou-Shady M., Zimmermann A., Lander A.D., Korc M., Kleeff J. and Büchler M.W. Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut. 48: 558-564, 2001.

27- Capurro M., Wanless I.R. and Sherman M. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology. 125: 89-97, 2003.

28- Sham J.G., Kievit F.M., Grierson J.R., Miyaoka R.S., Yeh M.M. and Zhang M. Glypican-3-targeted 89Zr PET imaging of hepatocellular carcinoma. J Nucl Med. 55 (5): 799-804, 2014.

29- Wu Y., Liu H. and Ding H. GPC-3 in hepatocellular carcinoma: current perspectives. J Hepatocell Carcinoma. 3:63-67, 2016.

30- Giordano S. and Columbano A. MicroRNAs: New tools for diagnosis, prognosis, and therapy in hepatocellular carcinoma? Hepatology. 57: 840-847, 2013.

31- Traister A., Shi W. and Filmus J. Mammalian Notum induces the release of glypicans and other GPI-anchored proteins from the cell surface. Biochem J. 15: 410 (3): 503-511, 2008.

32- Qiao S.S. Cui Z.Q., Gong L., Han H., Chen P.C., Guo L.M., Yu X., Wei Y.H., Ha S.A., Kim J.W., Jin Z.T., Li S., Peng J.R. and Leng XS. Simultaneous measurements of serum AFP, GPC-3 and HCCR for diagnosing hepatocellular carcinoma. Hepatogastroenterology. 58 (110-111): 1718-1724, 2011.

33- Chen M., Li G., Jian Y. and Hong T. Reevaluation of glypican-3 as a serological marker for HCC. Clin Chim Acta. 423:105-111, 2013.

34- Li L., Jin R. and Zhang X. Oncogenic activation of GPC3 by c-Myc in human HCC. Hepatology. 56 (4):1380-1390, 2012.

35- Capurro M.I., Xiang Y.Y., Lobe C. and Filmus J. Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res. 65 (14): 6245-6254, 2005.

36- Zittermann S.I., Capurro M.I., Shi W. and Filmus J. Soluble glypican 3 inhibits the growth of hepatocellular carcinoma in vitro and in vivo. Int J Cancer. 126 (6):1291-1301, 2010.

37- Sun C.K., Chua M.S., He J. and So S.K. Suppression of glypican 3 inhibits growth of hepatocellular carcinoma cells through up-regulation of TGF-β2. Neoplasia. 13 (8): 735-747, 2011.

38- Abd El-Gawad I.A., Ghada I.M., Noha H.R., Heba M.E. and Niveen M.E. Can Glypican 3 be Diagnostic for Early Hepatocellular Carcinoma among Egyptian Patients. Asian Pac. J. Cancer Prev. 14 (12): 7345-7349, 2013.

39- Hsu P.I. , Chow N.H., Lai K.H., Yang H.B., Chan S.H., Lin X.Z., Cheng J.S., Huang J.S., Ger L.P., Huang S.M., Yen M.Y. and Yang Y.F. Implications of serum basic fibroblast growth factor levels in chronic liver diseases and HCC. Anticancer Research. 17: 2803-2809, 1997.

40- Yamauchi N., Watanabe A. and Hishinuma M. The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. Mod Pathol. 18:1591-1598, 2005.

41- Yan D., He Q., Chen Y., Wang L. and Zhang X. Detection of α-fetoprotein and glypican-3 mRNAs in the peripheral blood of hepatocellular carcinoma patients by using multiple FQ-RT-PCR. J Clin Lab Anal. 25 (2): 113-117, 2011.

42- Xu C.I., Yan Z., Zhou L. and Wang Y. A comparison of GPC3 with AFP as a serum marker for hepatocellular carcinoma: a meta-analysis. J Cancer Res Clin Oncol. 139 (8):1417-1424, 2013.

43- Jia X., Liu J., Gao Y., Huang Y. and Du Z. Diagnosis accuracy of serum glypican-3 in patients with hepatocellular carcinoma: a systematic review with meta-analysis. Arch Med Res. 45 (7): 580-588, 2014.