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GPC3 gene expression and allelic discrimination of FZD7 gene in Egyptian patients with hepatocellular carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide, and especially in Egypt. Early diagnosis of HCC greatly improves the survival and prognosis of patients. Low sensitivity and specificity of alpha-fetoprotein (AFP) has led to the demand for novel biomarkers of HCC. The aim of the present study was to evaluate the validity of frizzled-7 (FZD7) and glypican-3 (GPC3) gene expression as potential biomarkers for HCC early diagnosis, and to investigate the association between FZD7 rs2280509 polymorphism and HCC risk.

Materials and methods: Quantification of FZD7 and GPC3 gene expression by real-

time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay, and genotyping FZD 7 (rs2280509 SNP) gene polymorphism using RT-PCR.

Results: The current results revealed that FZD7 gene expression had a greater area under the curve (AUC) for identifying HCC than GPC3 gene expression and AFP levels. The combination of the three markers as a panel showed a better diagnostic performance with a greater AUC than any of the single markers alone ($p < 0.05$). The FZD7 rs2280509 polymorphism (CT) was found to be significantly associated with an increased risk of HCC. The CT genotype and T allele were significantly more prevalent in the HCC group compared to either the cirrhosis ($p = 0.03$) or control groups ($p = 0.0009$ and 0.002; respectively).

Conclusion: FZD7 and GPC3 gene expressions have a complementary role in early HCC detection, with a greater diagnostic sensitivity and accuracy than AFP. In addition, FZD7 rs2280509 polymorphism is significantly associated with an increased risk of HCC in the Egyptian population.

Key words: HCC; frizzled-7; glypican-3; early detection

Introduction

Early detection of HCC increases the chance of treatment and improves prognosis [1]. The overall alpha-fetoprotein (AFP) performance has been unsatisfactory in terms of its poor sensitivity and specificity [2]; therefore, new markers with sufficient sensitivity and specificity are needed. Glypican-3 (GPC3) is a cell surface-bound proteoglycan that is expressed by most hepatocellular carcinomas (HCCs) [3]. It is highly expressed in AFP-negative HCC patients and is suggested to be a more sensitive marker than AFP for the detection of smaller HCC, with diameter of 3 cm or less [4]. GPC3 stimulates HCC growth by promoting canonical Wnt signaling. In addition to interacting with Wnt, GPC3 directly interacts with its signaling receptor Frizzled through the glycosaminoglycan chains of GPC3, indicating that this glypican stimulates the formation of signaling complexes between Wnt and Frizzled [3]. Frizzled7 is 1 of 10 members of the Frizzled family and the most studied member of this family and seems to be the most important Wnt receptor involved in cancer development and progression. FZD7 is the only frizzled receptor reported in HCC that is up-regulated in 90% of human HCCs [5, 6].

The aim of the present study was to evaluate the validity of FZD7 and GPC3 gene expression as potential biomarkers for HCC early diagnosis, and to investigate the association between FZD 7 rs2280509 polymorphism and HCC.

Materials and methods

Study groups

The current study was conducted on 164 subjects selected from the National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt, in the interval from December 2016 to April 2017. A written informed consent was taken from all patients selected to participate in the study.

The study groups were divided as follows:

- Group 1: 50 normal individuals as control group;
- Group 2: 57 cirrhotic patients infected with chronic hepatitis C virus (HCV) genotype-4, newly diagnosed on the basis of history, clinical examination, laboratory findings and US assessment;
- Group 3: 57 hepatocellular carcinoma patients proven to be infected with chronic HCV genotype-4 by PCR, diagnosed by ultrasonography (US) assessment, abdominal triphasic computed tomography (CT) and serum AFP.

Selection of patients

Inclusion criteria of control group were as follows:

- adult males or females (19–77 years old);
- negative serum HCV Antibody by ELISA;
- negative serum hepatitis B virus (HBV) surface antigen by ELISA;
- normal serum aminotransferases [aspartate transferase (AST) and alanine aminotransferase (ALT)] levels.

Exclusion criteria of patients group were as follows:

Patients with severe heart or brain disease, viral disease other than HCV or HBV infection or other malignant tumors.

Clinical assessment

Full medical history was taken with special reference to risk factors of liver diseases

Physical examination included abdominal pain, weight loss, jaundice, abdominal enlargement, vomiting of blood, bleeding and elevation of body temperature.

Sample collection and measurement of tumor markers: Venous blood samples were collected by vein puncture from all subjects included in the study, and then hematological (hemoglobin, and platelet count), and biochemical analyses (liver functions; AST, ALT, total bilirubin, serum albumin and prothrombin time, blood glucose and serum creatinine), were carried out.

Quantitative determination of serum alpha-fetoprotein (AFP) was carried out using the CanAg AFP EIA kit that is intended for the quantitative determination of AFP concentration in human serum (Cat.No. 600-10, Fujire Bio Diagnostics, Inc.). Quantification of FZD 7 and GPC3 gene expression was done by by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay which first included preparation and isolation of peripheral blood mononuclear cells (PBMCs), then extraction and purification of RNA from PBMCs cells **[**7] using the RNeasy Mini Kit (Qiagen, Germany) (Cat. no. 74104), and then the concentration and purity of total RNA were calculated by measuring the absorbance at 260 and 280 nm using (Nano Drop 2000 spectrophotometer, Thermo Scientific, Unietd States). Synthesis of complementary DNA (cDNA) was then performed according to the method of [8] using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, United States). The relative quantitative defection of FZD7 and GPC3 gene expressions were performed using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, United States).All samples were analyzed using the StepOne Real-Time PCR Analyzer (Applied BioSystems, California, United States). The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression determined from qRT-PCR experiments [9].

Genotyping for the Frizzled 7 (rs2280509 SNP) gene polymorphism using RT-PCR

Extraction of genomic DNA from PBMC was achieved using QIAamp DNA Mini kit (Qiagen, Germany) (Cat. No. 51304), following the manufacturer's instructions, and then FZD7 gene polymorphism rs2280509 was analyzed using specific primers and Taqman probes (Taqman®SNP Genotyping Assay Kit, Applied Biosystems, Foster City, CA, United States).

Genotyping of FZD7 gene polymorphism rs2280509 was achieved by the allelic discrimination Real-Time PCR system using dual labeled fluorogenicTaqMan® minor groove binder (MGB) probes with Sequence-specific forward and reverse primers pre-designed to amplify the sequence containing the SNP (Applied Biosystems, Carlsbad, California, United States) according to the manufacturer's instructions.

Statistical analyses

SPSS for windows (version 20.0) was used. Statistical evaluation of the data was performed by one-way ANOVA, Mann-Whitney U test, ROC curve and Chi-square test.

Results

The demographic characteristics, biochemical and hematological data of the studied patients and controls are summarized in Tables 1 and 2, respectively. HCC patients showed a significantly higher mean values of ALT, AST, total bilirubin, creatinine, glucose and international normalized ratio (INR), with a significantly lower mean values of haemoglobin (Hb) ($p < 0.001$) compared to the control group.

Table 1. Demographic characteristics of the studied groups

Groups **Variable**	**Control** **(n = 50)**	**LC** **(n = 57)**	**HCC** **(n = 57)**
Gender			
Male, No. (%)	33 (66%)	33 (57.9%)	39 (68.4%)
Female, No. (%)	17 (34%)	24 (42.1%)	18 (31.6 %)
Age [Years]			
Range	19–77	28–80	48–89
Mean ± SD	44.02 ± 16.84	54.44[a] ± 9.79	60.19[a] ± 8.48
BMI [kg/m^2]			
Range	18–30	22.6–35	20–32
Mean ± SD	22.26 ± 3.55	26.56 [a] ± 2.83	26.16 [a] ± 2.11
Smoking (%)			
Yes	6 (12%)	11(19.3%)	35(61.4%) [a]
No	44 (88%)	46 (80.7%)	22 (38.6%)

Alcohol intake (%)			
Yes	4 (8%)	0 (0%)	1 (1.8%)
No	46 (92%)	57 (100%)	56 (98.2%)

p-values < 0.05 were considered significant. aStatistically highly significant compared to normal controls, p-value < 0.001. SD — standard deviation. LC — liver cirrhosis; HCC — hepatocellular carcinoma

Table 2. Biochemical and hematological parameters of all studied groups

HCC (n = 57)	LC (n = 57)	Control (n = 50)	%D		Variables Means ± SD
			HCC	LC	
62.52[a]± 32.18	50.14[b] ± 25.17	5.70 ± 30.34	106.1	65.3	ALT [U/L]
104.87[a]± 62.35	77.0[b] ± 46.47	7.48 ± 33.28	215.1	131.4	AST [U/L]
4.38[a]± 3.79	4.75[a]± 3.85	0.19 ± 0.77	468.8	516.9	T.Bil [mg/dl]
2.65[a]± 0.52	2.51[a]± 0.84	0.21 ± 3.84	-31	-34.6	Albumin [g/dl]
2.1[a]± 1.70	[b] ± 1.17 1.56	0.16 ± 0.96	118.7	62.5	Creatinine [mg/dl]
221.1[a]± 117.5	207.1[a]± 79.6	±15.3 103.1	114.5	100.8	Glucose [mg/dl]
1.46[a]± 0.29	1.49[a]± 0.71	0.06 ± 0.98	49	52.04	INR
114.9[a]± 48.3	141.6[a]± 71.7	±88.8 284.3	59.6-	-50.2	**platelet [10^9/L]**
1.63 11.28±	10.68[b] ± 2.12	1.51 ± 11.94	-5.5	10.5-	**Hb [g/dL]**
3.98 7.43±	5.38 7.84±	2.01 ± 7.20	3.2	8.9	**WBCs [10^9/L]**

Data are represented as mean ± standard deviation (SD). Statistical analyses were carried out using one way analysis of variance (ANOVA) and Tukey's multiple comparisons test. a: statistically highly significant compared to normal controls, p-value < 0.001; b: statistically significant compared to normal controls, p-value < 0.01. p-values < 0.05 were considered significant. D%: percentage difference compared to normal controls. LC — liver cirrhosis; HCC — hepatocellular carcinoma; ALT — alanine aminotransferase; AST — aspartate transferase; T.Bil — total bilirubin; INR — international normalized ratio; Hb —haemoglobin; WBCs — white blood cells

In the present study, the median serum AFP level in the LC and HCC groups was significantly higher ($p < 0.001$) than that in the control group with no significant difference between the HCC and LC groups ($p = 0.18$). On the other hand, comparing the median expression value of FZD7 between the studied groups revealed a high statistically significant difference ($p < 0.001$) between the control group (32) and both the LC group (512), and the HCC group (1024), along with a significant difference ($p < 0.01$) between the LC group and the HCC group. Moreover, the median values of GPC3 gene expression have revealed a high statistically significant difference ($p < 0.001$) between the control group (32) and both the LC group (256), and the HCC group (512), along with a high significant difference ($p < 0.001$) between the LC group and the HCC group (Tab. 3).

Table 3. Levels of serum alpha-fetoprotein (AFP) along with glypican-3 (GPC3) and frizzled-7 (FZD7) gene expressions in the studied groups

Group **Parameter**	**Control** **(n = 50)**	**LC** **(n = 57)**	**HCC** **(n = 57)**
[AFP [µg/L **Median**	6	22	93
IQR	2	318	530
			p-value
Control	–	< 0.001**	< 0.001**
LC	**0.001 >	–	0.18
HCC	< 0.001**	0.18	–
FZD7 gene expression ($2^{-\Delta\Delta CT}$) **Median**	32	512	1024
IQR	66	896	1536
			p-value
Control	–	< 0.001**	< 0.001**
LC	**0.001 >	–	*0.01 >
HCC	< 0.001**	*0.01 >	–
GPC3 gene expression ($2^{-\Delta\Delta CT}$) **Median**	32	256	512
IQR	56	448	1280
			p-value
Control	–	< 0.001**	< 0.001**
LC	**0.001 >	–	**0.001 >
HCC	< 0.001**	**0.001 >	–

Data are represented as median and interquartile range (IQR). Statistical analysis was carried out using Mann-Whitney U test. Relative expression levels of FZD7 and GPC3 results were analyzed using the 2−ΔΔCTmethod. p-values < 0.05 were considered significant.*p < 0.01; significant, **p < 0.001; highly significant. LC — liver cirrhosis; HCC — hepatocellular carcinoma

Spearman's correlation analysis revealed a significant positive correlation between FZD7 and GPC3 gene expression levels (r =0.344) in HCC patients. However, no

significant correlation could be found between AFP levels and either FZD7 or GPC3 gene expression levels (Tab. 4).

Table 4. Spearman's correlation analysis of serum alpha-fetoprotein (AFP), glypican-3 (GPC3) and frizzled-7 (FZD7) gene expressions in the studied groups

Parameter	**FZD7 gene expression**	**GPC3 gene expression**	**Serum AFP concentration**
FZD7 gene expression			
r	1	0.344	–0.123
p		0.009*	NS
GPC3 gene expression			
r	0.344	1	–0.06
p	0.009*		NS
Serum AFP concentration			
r	–0.123	–0.06	1
p	NS	NS	

r (+) = Positive correlation; r (-) = Negative correlation, *p < 0.01; significant; NS — not significant

A highly significant positive correlation was found between FZD7 gene expression levels and tumor size ≥ 5 cm (r = 0.493). Meanwhile, no significant correlations could be found between GPC3 gene expression and tumor sizes ≥ 5 cm, > 3 or ≤ 3 cm (Tab. 5).

Table 5. Spearman's correlation analysis for the expression levels of frizzled-7 (FZD7) and glypican-3 (GPC3) along with tumor size

Parameter	**Tumor size [cm]**		
	≤ 3	**> 3**	**≥ 5**
FZD7 expression levels			
r-value	–0.198	0.198	0.493
p	NS	NS	**0.001
GPC3 expression levels			

0.106	0.202	–0.202	r-value
NS	NS	NS	p

r (+) = positive correlation; r (–) — negative correlation; **p < 0.001; highly significant; NS — not significant

The genotype frequency of FZD7 rs2280509 polymorphism was in compliance with the Hardy Weinberg equilibrium (HWE) in both the control group and the cirrhosis group (*p*> 0.05), while a deviation from HWE was observed in the HCC group (p < 0.05). The frequency distribution of the different genotypes and alleles for FZD7 gene polymorphism is shown in Table 6. There was a statistically significant difference between HCC patients and each of the other groups. **To evaluate the risk of HCC according to the FZD7 rs2280509 genotype using the wild type CC genotype as the reference genotype [The sentence seems incomplete, please verify and consider rephrasing]** (http://www.ncbi.nlm.nih.gov/projects/SNP). The CT genotype and T allele were significantly more prevalent in the HCC compared to either the cirrhosis or control groups. Whereas the CC genotype and C allele were significantly more prevalent in the control group.

Compared to the control group, the prevalence of CT genotype and the combined CT + TT genotypes showed a significant difference between HCC group and control group [odds ratio (OR) = 3.929, p = 0.0009 & OR = 4.0, p = 0.0006; respectively). In addition, the T allele was associated with higher risk of HCC development (OR = 2.730, p= 0.002).

Table 6. Frizzled-7 (FZD7) genotype and allele frequency distribution in the studied groups

	Control (n = 50)	**LC (n = 57)**	**HCC (n = 57)**	**HCC *vs.* LCs**		**HCC *vs.* Control**		**LC *vs.* Control**	
				p-value	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**
FZD7 genotype distribution, N (%)									

TT	1 (2%)	5 (8.8%)	3 (5.3%)	0.876	0.885 (0.216 – 4.010)	0.139	5.000 (0.688 – 66.46)	0.088	5.645 (0.678 – 68.13)
CT	14 (28%)	21 (36.8%)	33 (57.9%)	0.03*	2.320 (1.076 – 4.823)	0.0009***	3.929 (1.695 – 9.018)	0.212	1.694 (0.767–3.981)
CC	35 (70%)	31 (54.4%)	21 (36.8%)	Reference genotype (wild type)		–		–	
HWE (p-value)	0.768	0.599	0.031						
Genotypes									
CC	35 (70%)	31 (54.4%)	21 (36.8%)	0.060	2.044 (0.975 – 4.352)	0.0006***	4.000 (1.781 – 9.098)	0.097	1.957 (0.876 – 4.294)
CT+TT	15 (30%)	26 (45.6%)	36 (63.2%)						
Allele Frequency distribution, N (%)									
T	16 (16%)	31 (27.2%)	39 (34.2%)	0.251	1.392 (0.780 – 2.421)	0.002**	2.730 (1.444 – 5.150)	0.04*	1.961 (0.996 – 3.729)
C	84 (84%)	83 (72.8%)	75 (65.8%)	Reference genotype (wild type)		–		–	

Data are expressed as number and percentage, n (%). *, ** and *** represent significant difference at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Odds ratio (OR) with 95% confidence intervals (CIs) test were used. (OR = 1, indicating that the factor does not influence the occurrence of the disease; OR > 1, indicating that the factor is a risk factor; OR < 1, indicating that the factor is a protective factor). LC — liver cirrhosis; HCC — hepatocellular carcinoma; HWE — Hardy Weinberg equilibrium

The high OR indicates the strength of association between the studied locus and the disease. Adverse alleles were defined as the minor allele of the risk-affect SNPs (OR >

1) and the common allele of the protective-effect SNPs (OR < 1). $P_{HWE} > 0.05$ indicated a consistent with HWE) or ($P_{HWE} < 0.05$ indicated a deviation from HWE).

No significant association could be detected between FZD7 rs2280509 polymorphism or allele and its gene expression (p = 0.061, p = 0.051; respectively). However, CC/C carriers had significantly higher FZD7 expression levels compared to the TT/T carriers, while the CT had significantly lower FZD7 expression levels. Meanwhile, the combined CT + TT genotypes had significantly lower FZD7 expression levels than the CC genotype (p = 0.019) (Tab. 7).

Table 7. Association between frizzled-7 (FZD7) gene polymorphism and FZD7 gene expression levels

	HCC group (n = 57)		
	FZD7 expression (median value = 1024)		χ^2 **p-value**
	High expression (≥ 1024) (n = 32)	**Low expression (< 1024) (n = 25)**	
FZD7 gene polymorphism (rs2280509)			
CC (n = 21)	16 (50%)	5 (20%)	5.593 p = 0.061
CT(n = 33)	15 (46.8%)	18 (72%)	
TT (n = 3)	1(3.1%)	2(8%)	
Polymorphism			
CC	(50%) 16	(20%)5	5.429 p = 0.019*
CT+TT	(50%) 16	20 (80%)	
Allele			
C	(73.4) 47	(56) 28	3.792 p = 0.051
T	(26.6) 17	(44%)22	

Data are expressed as number and percentage, n (%); Statistical analyses were carried out using Chi-square test ($\chi 2$). $\chi 2$ is significant at $p < 0.05$, *$p < 0.01$; significant. HCC — hepatocellular carcinoma

From ROC curve analysis, FZD7 had a greater AUC for identifying HCC than either GPC3 or AFP (AUC = 0.820 vs. 0.756 and 0.709, respectively). The sensitivity and

specificity of single AFP detection was 65% and 73%, respectively, and the sensitivity and specificity of single GPC3 detection was 77% and 72%, respectively; while the sensitivity and specificity of single FZD7 detection was 86% and 64%, respectively (Tab. 8). On the other hand, the sensitivity and specificity of combined GPC3 + AFP detection reached 84% and 72%, respectively, the sensitivity and specificity of combined FZD7 + AFP detection reached 95% and 61%, respectively; while the sensitivity and specificity of combined GPC3+FZD7 detection was 79% and 76%, respectively (Tab. 9). Consequently, the results of the ROC curve analysis suggested that the combination of AFP with GPC3 or the combination of AFP with FZD7 could increase the diagnostic accuracy of AFP with greater AUC in the diagnosis of HCC (Fig. 1).

Table 8. Diagnostic accuracy of frizzled-7 (FZD7), glypican-3 (GPC3) and alpha-fetoprotein (AFP) according to receiver operating characteristic (ROC) curve analysis

Marker	**AFP [µg/L]**	**GPC3 gene expression ($2^{-\Delta\Delta CT}$)**	**FZD7 gene expression ($2^{-\Delta\Delta CT}$)**
Cut off	21	192	192
AUC	0.709	0.756	0.820
95% CI	0.620 - 0.797	0.736 - 0.874	0.758 - 0.882
Sensitivity (%)	65	77	86
Specificity (%)	73	72	64
PPV (%)	56.1	59.5	55.6
NPV (%)	79.6	85.5	89.5
FDR (%)	43.9%	40.5%	44.4%
Accuracy (%)	70	73.7	71.3
p-value	< 0.001**	< 0.001**	< 0.001**

Statistical significance,**p < 0.001; highly significant**;** AUC — area under the curve**;** CI — confidence interval; PPV — positive predictive values; NPV — negative predictive values; FDR — false discovery rate

Table 9. Combined analysis of alpha-fetoprotein (AFP), glypican-3 (GPC3) and frizzled-7 (FZD7), for hepatocellular carcinoma (HCC) diagnosis according to to receiver operating characteristic (ROC) curve analysis

Marker	**AFP + GPC3**	**AFP + FZD7**	**GPC3 +FZD7**
AUC	0.853	0.845	0.851

95% CI	0.795 - 0.910	0.788–0.903	0.792–0.910
Sensitivity (%)	84	95	79
Specificity (%)	72	61	76
PPV	61.5	56.3	63.4
NPV	89.5	95.5	87
Accuracy (%)	76.2	72.5	76.8
p-value	< 0.001**	< 0.001**	< 0.001**

Statistical significance, **p < 0.001; highly significant; AUC — area under the curve; CI — confidence interval; PPV — positive predictive values; NPV — negative predictive values

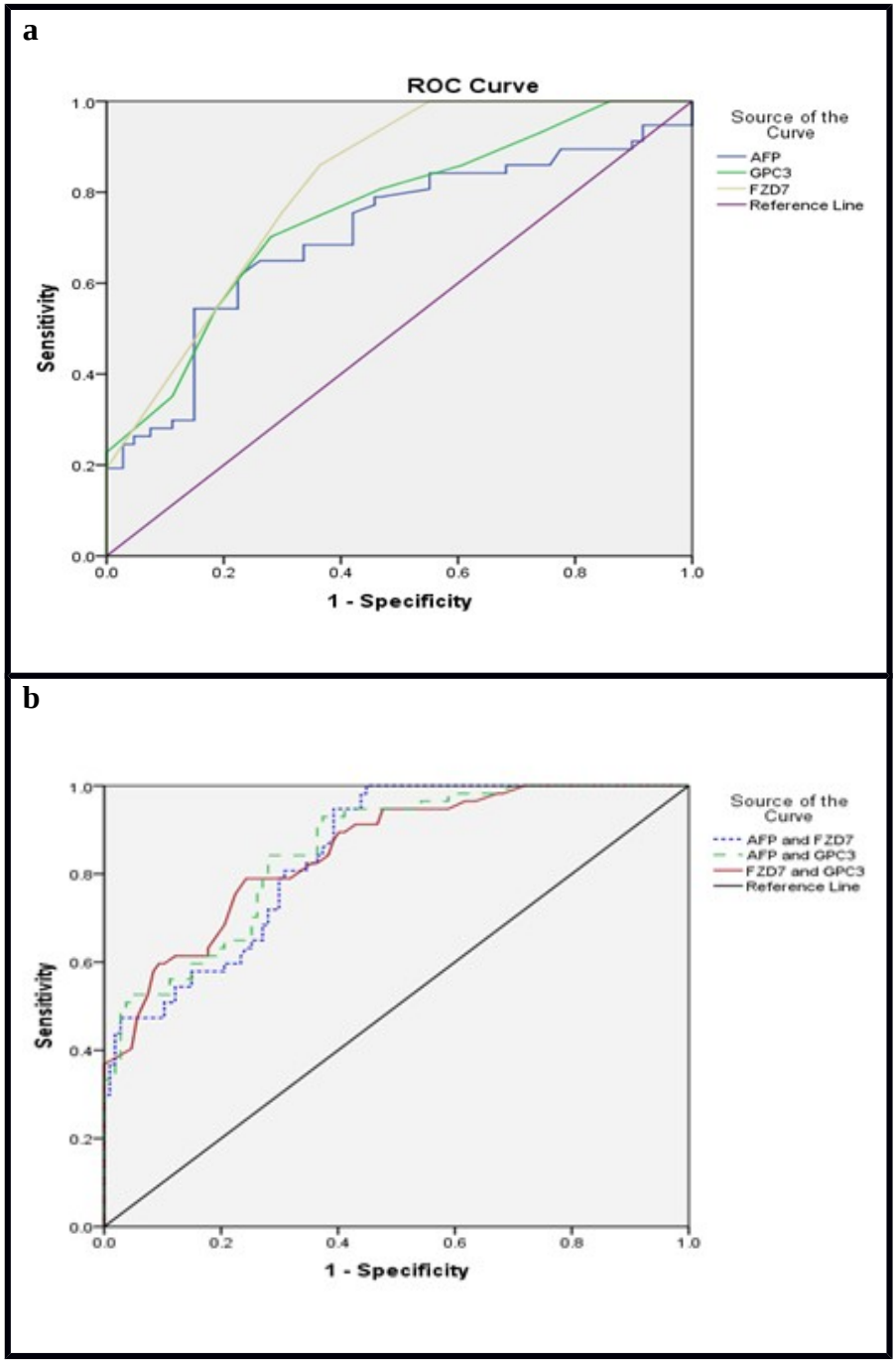

Figure 1A. Receiver operating characteristic (ROC) Curve of frizzled-7 (FZD7), glypican-3 (GPC3), and alpha-fetoprotein (AFP) for hepatocellular carcinoma (HCC) prediction vs. other groups; **B.** ROC Curve for combined analysis of HCC tumor markers

Discussion

The current study aimed to validate the efficacy of GPC3 and FZD7 gene expression in early diagnosis of HCC as compared to AFP. The median value of serum AFP was significantly higher in the HCC group (93 µg/L) ($p < 0.001$) compared to both liver cirrhosis (LC) and normal control (NC) groups (22 µg/L and 6 µg/L, respectively).

The median expression level of GPC3 was found to be significantly ($p < 0.001$) higher in the HCC group (512) compared to both the LC group (256) and the normal control group (32), which is in agreement with [10–13]. The median expression level of FZD7 gene expression was also found to be significantly higher ($p < 0.001$) in the HCC group (1024) compared to both the LC group (512) and the normal control group (32).

However, when ROC curve analysis was applied for the HCC group compared to the LC and NC groups, the AFP cut-off value was 21 µg/L which gave an overall sensitivity and specificity of only 65% and 73%, respectively, with almost 35% of the HCC patients being negatively diagnosed which confirms that the performance of AFP is only suboptimal [14].

These results are in accordance with [15] who assessed an AFP cut off value > 20 ng/mL, which gave only 61% sensitivity and 71% specificity. Several studies have shown that the detection efficacy of AFP for early stage HCC varies considerably and, at high levels, its sensitivity is within the 39–65% range and specificity within the 76–94% range [16].

Meanwhile, GPC3 cut-off value was 192, which gave 77% sensitivity and 72% specificity, with almost only 23% of the HCC patients being negatively diagnosed. These results are in agreement with Yao et al., 2013 [17], who reported that the sensitivity and specificity of GPC3 for differentiating HCC from non-malignant

neoplastic nodules were 75.4% and 72.4%, respectively**.** These results are also in agreement with Feng and Ho [18] who detected a higher sensitivity of GPC3 expression levels than serum AFP level (71.7% *vs.* 51.3%) in early detection of HCC **[35].** Previous studies reported that the sensitivity of GPC3 for HCC ranges from 75% to 100%, and in large-scale trials it ranged from 75% to 85% [19–21]. Libbrecht et al. [22] found that the sensitivity of a positive GPC3-staining for the diagnosis of HCC in small focal lesions was 77%. On the other hand, ROC curve analysis for the FZD7 gene expression levels revealed the cut-off value to be 192 which gave 86% sensitivity and 64% specificity, with only 14% of HCC patients being negatively diagnosed and, again, FZD7 gene expression alone (AUC = 0.820, sensitivity 86%) or in combination with AFP (AUC = 0.845, sensitivity 95%), was better than AFP alone in the diagnosis of HCC. Combining the two markers AFP and GPC3 expression had a better AUC of 0.853, sensitivity of 84%, and specificity of 72%. These results are in agreement with a previous study [13] which concluded that GPC3, alone or in combination with AFP, was better than AFP alone in the diagnosis of HCC. The AUC of combining AFP and GPC3 was 0.898 with 85% sensitivity, greater than AFP alone (AUC = 0.754, sensitivity 68%) or GPC3 alone (AUC =0.850, sensitivity 78.7%) [19]. These results are also in agreement with Xu et al. [23] who found that the combination of GPC3 and AFP yielded a better sensitivity (85%) and AUC (0.936) than either of them alone in the differential diagnosis of HCC. However, the specificity of GPC3+AFP was lower than that of AFP alone. Moreover, combining the two markers FZD7 expression and AFP had a better AUC of 0.845, which recorded sensitivity of 95%, and specificity of 61%, while combining the two markers GPC3 and FZD7 expression had an AUC of 0.851, sensitivity of 79%, and specificity of 76%. Consequently, the combination of AFP with GPC3 or the combination of AFP with FZD7 could both increase the diagnostic capacity of AFP with greater AUC. Future large-scale association studies are needed to validate the current results and define the functional effects of FZD7 gene expression on HCC progression and also the effectiveness of FZD7 alone or in combined analysis for HCC diagnosis**.**

Spearman's correlation analysis revealed a highly significant positive correlation ($r = 0.493$, $p < 0.001$) between FZD7 gene expression levels and tumor size ≥ 5 cm. The same finding was applicable for tumor size less than 5 cm (> 3 and ≤ 3cm). On the other

hand, Spearman's correlation analysis revealed a significant positive correlation between FZD7 and GPC3 gene expression levels in HCC patients ($r = 0.344$, $p = 0.009$). However, Spearman's correlation analysis did not reveal any correlation between GPC3 expression levels and tumor sizes (≥ 3; < 3 cm or ≥ 5 cm). The current results are in agreement with several previous studies [11, 24, 25, 26]. The current results are also supported by other studies which did not find any significant correlation between GPC3 gene expression and tumor size ≥ 5cm [24, 27–30].

FZD7 rs2280509 polymorphism and HCC:

The CT genotype (57.9%) and T allele (34.2%) were significantly more prevalent in the HCC group compared to either the cirrhosis group ($p = 0.03$ and $p = 0.251$, respectively) or control group ($p = 0.0009$ and $p = 0.002$, respectively). Meanwhile, the CC genotype and C allele were significantly (70% and 84%) more prevalent in the control group, indicating that the FZD7 rs2280509 CT genotype may be a potential risk factor for HCC. The P_{HWE} in the HCC group ($p = 0.031$) that indicated a deviation from $P_{HWE} < 0.05$. The deviation from HWE in the HCC group may be indicative of association with the disease [31]. Compared to the control group, prevalence of CT genotype and the combined CT + TT genotypes showed a significant difference between HCC group and control group (OR = 3.929, $p = 0.0009$ & OR = 4.0, $p = 0.0006$; respectively). In addition, the T allele was associated with a higher risk of HCC development (OR = 2.730, $p = 0.002$).

The study did not reveal any significant correlation between FZD7 gene expression and either its genotype frequency ($p = 0.061$) or its allele frequency ($p = 0.051$). However, CC/C carriers had significantly higher FZD7 expression levels compared to the TT/T carriers, while the CT had significantly lower FZD7 expression levels. Meanwhile, the combined CT + TT genotypes had significantly lower FZD7 expression levels than the CC genotype ($p = 0.019$). Therefore, it could be hypothesized that single nucleotide polymorphisms (SNP) in FZD7 gene might affect its expression or function which might have an impact on the development of HCC. Further studies on larger sample size and samples from other geographical regions are needed to examine the associations between FZD7 C/T polymorphism and HCC risk.

Conclusion

In summary, the findings of the present study suggested that FZD7 rs2280509 polymorphism is associated with an increased risk of HCC in the Egyptian population.

FZD7, GPC3 and AFP have a complementary role in HCC detection. FZD7 gene expression and GPC3 gene expression increase the diagnostic yield in AFP-negative HCC and have greater diagnostic sensitivity and accuracy than AFP in the diagnosis of HCC. Additionally, GPC3 and FZD7 have promising roles in the pre-clinical diagnosis of HCC.

Finally, according to the current results it can be concluded that FZD7 gene expression has a better sensitivity than AFP for the diagnosis of HCC. Current evidence indicates that FZD7, GPC3 with AFP exhibit much better sensitivity for the diagnosis of HCC when used in combination rather than alone. Further studies on larger samples from different geographic regions are needed to explore the association between FZD7 gene polymorphism and HCC.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards**.**

Ethical approval was waived by the local Ethics Committee of Ain Shams University in view of the retrospective nature of the study and all the procedures being performed as part of the routine care

Conflict of interests

The authors have no relevant financial or non-financial interests to disclose.

The authors have no competing interests to declare that are relevant to the content of this article.

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