

# New Genetic Markers for Diagnosis of Hepatitis C Related Hepatocellular Carcinoma in Egyptian Patients

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## ABSTRACT

**Background and aim:** Early detection of hepatocellular carcinoma (HCC) enhances effective and curative management. New genetic markers with distinct diagnostic ability are required. **Aim:** determine the expression of GPC3, PEG10, SERPINI1, MK and QP-C in the peripheral blood of HCC patients.

**Methods:** 74 HCV patients were recruited and divided into three groups; chronic hepatitis (I), liver cirrhosis (II) and HCC (III). Demographics, laboratory and imaging data were collected. Child score and metastatic work up were completed. The expression of the five candidate genes in the peripheral blood was performed by qRT-PCR assay.

**Results:** Groups were gender matched, age in group I was significantly lower than in groups II and III (37.7 vs 50.4 and 55.6, p value <0.005). CHILD score; group II and III A/B/C = (7/5/6) and (20/6/3). AFP was significantly higher in group III than I and II (204 vs 3.9 and 6.9, p < 0.01). In HCC group 69% of the lesions were < 5 cm, and had 1-2 nodules; 14% had metastases. GPC3, PEG10, SERPINI1 and MK mRNA were significantly higher in the HCC group compared to the other groups while QP-C mRNA was higher in chronic hepatitis C group compared to other groups. The gene expression values in HCC patients were independent of the tumor size, AFP levels or extrahepatic metastasis. Combined measurement of the five gene markers showed 100% sensitivity and 33% specificity, 48% PPV and 100% NPV.

**Conclusion:** GPC3, PEG10, SERPINI1 and MK are genetic markers that can represent a useful tool for detection of HCC.

**Key words:** GPC3 – PEG10 – SERPINI1 – MK – QP-C – hepatocellular carcinoma – gene markers.

## INTRODUCTION

Diagnosis of hepatocellular carcinoma (HCC) was considered a terminal situation and the leading cause of death in cirrhotic patients [1]. Hepatocellular carcinoma is the seventh most common cancer worldwide, and the third leading cause of cancer-related deaths [2]. However, when diagnosis is achieved at an early stage, effective therapies that improve long term survival can be applied [3].

In Egypt, HCC was reported to account for about 4.7% of chronic liver disease patients [4] with a doubling in the incidence rate in the past 10 years [5]. The

HCC epidemic in Egypt is associated with hepatitis C viral infection (HCV); Egypt has the highest prevalence of HCV in the world with ~13.8% of the population infected and seven million persons with chronic HCV liver disease [6].

Since its detection in the serum of HCC patients in 1970s, alpha-fetoprotein (AFP) has been the only serologic marker widely used for diagnosing HCC patients [7]. As a limitation, elevated serum AFP is observed in only 60% to 70% of HCC patients and, to a lesser extent (33-65%) in patients with smaller HCCs [8]. Moreover, nonspecific elevation of serum AFP has been found in some patients with chronic hepatitis and patients with liver cirrhosis. Therefore, it was necessary to identify new HCC markers that have a sufficient sensitivity and specificity for the diagnosis of HCC patients, especially in AFP-normal and/or smaller HCC cases.

Glypican3 [GPC3], midkine [MDK], paternally expressed 10 [PEG10], serine protease inhibitor 11 [SERPINI1] and low molecular mass ubiquinone-binding protein [QP-C] are five genes that have been newly studied in HCC samples, including those with normal serum AFP and small tumors in Japanese patients [9], and revealed a significant increase in their

expression in most cases. This study showed that a combined score of these five genes can accurately classify non-cancerous hepatic tissues (100%) and HCC (71%).

Due to the complexity of the HCC situation in Egypt, and searching for an early and accurate diagnostic tool, we studied and evaluated the diagnostic significance of the five genes mRNA (GPC3, PEG10, MDK, SERPINI1 and QP-C) in the peripheral blood of Egyptian patients with HCC due to HCV genotype 4 (HCVG4) by means of a highly sensitive qRT-PCR assay.

## METHODS

### Population samples

This study was conducted on 74 patients with chronic HCVG4 infection being treated at the Faculty of Medicine, Cairo University. The patients were classified into 3 groups: group I chronic HCV hepatitis (27 patients HAI > A1, F<4 on liver biopsy by Metavir score), group II liver cirrhosis (18 patients post HCV cirrhosis on ultrasonography and F4 on liver biopsy by Metavir score) [10], group III HCC (29 patients diagnosed according to the guidelines of AASLD 2005) [11]. Exclusion criteria: HCC that underwent ablative therapy, patients who received interferon therapy and patients with liver cirrhosis of other etiologies rather than post-HCV. In addition, there were 7 healthy individuals who served as a control for the level of the five candidate genes. Informed consent was obtained from all participants before enrolment in the study. The study was performed in accordance with the principles of the Declaration of Helsinki, and its appendices, and with local and national laws.

All the patients were subjected to complete clinical assessment with special emphasis on the manifestation of liver cell failure and metastasis. Laboratory investigations were undertaken including a complete liver profile, international ratio (INR) and AFP with a reference normal level of the used kit 10 ng/ml using Axyam-Abbot (USA). Viral hepatitis markers were performed (HBsAg, HBc total Ab, HCV Ab by ELISA and HCV RNA by PCR). Abdominal ultrasound and color doppler were completed for all the enrolled patients and they were examined after at least 8 hours fasting. Child Pugh score and MELD score were calculated to identify the disease severity of cirrhotic patients. For patients with HCC lesions, BCLC staging [3] and metastatic work up (CT chest, bone scan) were undertaken. Percutaneous liver biopsy was performed under ultrasound guidance using 16 gauge needles and sent for histopathological examination for confirmation of the diagnosis, staging and exclusion of other causes. Ishak classification was used for the assessment of necroinflammation and stage of fibrosis in HCV cases [12], while histopathological evaluation of HCC cases were made according to Schaff et al [13]. The five candidate genes (GPC3, PEG10, MDK, SERPINI1 and QP-C) levels were evaluated by quantitative RT-PCR measured in peripheral blood mononuclear cells.

### Processing of samples

Two milliliters (ml) of venous blood was withdrawn from each subject. Blood was admitted in a 2 ml EDTA tube to study the gene expression. Blood samples were obtained from seven

disease-free liver donors to extract a pooled RNA sample to be used as calibrator for gene expression studied in peripheral blood mononuclear cells. For all participants, total human RNA is isolated using QIAamp RNA Blood Mini Kit (Qiagen Cat. No. 52304). The used reagents to measure the five genes were:

- TaqMan Universal PCR Master Mix with AmpErase UNG (2X) (Applied Biosystems P/N 4364438)
- 20X TaqMan Gene Expression Assay Mix for target genes (Applied Biosystems): Each includes 20X mix of unlabeled PCR forward and reverse primers and TaqMan® MGB probe (FAM™ dye-labeled).
  - GPC3: Hs00170471\_m1
  - MDK: Hs00171064\_m1
  - QP-C: Hs00429571\_g1
  - PEG10: Hs00248288\_s1
  - SERPINI1: Hs00192380\_m1
- TaqMan Ribosomal RNA control reagent (VIC Dye) (Applied Biosystems P/N 4308329).

### Statistical analysis

The descriptive statistics were provided with mean ± standard deviation (SD) or median for non parametric data. The  $\chi^2$  test and *t*-Student test were employed for the analysis of qualitative or quantitative variables, respectively. Pearson correlation was performed to correlate continuous variables, and Spearman correlation for correlating fibrosis stages with other variables. In all tests, *p* values were significant if less than 0.05.

## RESULTS

Comparative data of baseline features of 74 patients enrolled in this study are shown in Table I. Age is significantly lower in chronic hepatitis patients (group I) than in the liver cirrhosis (group II) and HCC (group III) patients; *p*=0.05, while gender showed no statistical significant difference. As regards the Child Pugh score (A/B/C) in group II and III, they were (7- 38.9% 5 - 27.8%, 6 - 33.3%) and (20 - 68.9%, 6 - 20.7%, 3 - 10.3%). In the assessment of the liver profile there was a significant increase of alkaline phosphatase and AFP in the HCC group (*p* <0.01) while chronic hepatitis patients showed significantly lower bilirubin level and INR (*p* <0.01).

GPC 3, PEG 10, MDK and SERPINI1 gene expressions were significantly higher in the HCC group compared to the liver cirrhosis and CHC group (*p*-0.022, 0.01, <0.01, <0.01, respectively). QP-C gene expression was higher in the CHC group. The results of gene expression values are expressed by a relative unit (target quantities were expressed as an *n*-fold difference relative to the calibrator that was obtained from pooled RNA extracted from the peripheral blood of healthy individuals) (Fig.1). Staging of HCC patients according to the Barcelona clinic liver cancer (BCLC) scoring system demonstrated that most of the patients were stage A or B, while the minority were stage C or D. Most of the HCC lesions (69%) were single or two nodules and 51.7% of the lesions were 3-5 cm size in the biggest diameters. Distant metastasis was present in only 13.8% of the lesions. Portal vein invasion was present in a single case. The HCC patients they were grouped according to AFP level into; AFP of <10, 10-200, and >200 ng/

**Table I.** Demographic, laboratory and genetic features of the studied groups

	Non HCC			P- Value
	Chronic HCV (N: 27)	Liver cirrhosis (N: 18)	HCC (N: 29)	
Age (mean± SD)	37.78 ± 11.4 B	50.4 ± 8.1 A	55.6 ± 7.9 A	0.05
Sex				
Male : Female	17:10 A	14:4 A	20: 9 A	0.58
Child Pugh Score		7	20	
Grade A		5	6	
Grade B		6	3	
Grade C				
AST (U/L)	43.3 ± 24.1 B	61.89 ± 27.5 A	56.8 ± 34.5 AB	0.093
ALT (U/L)	52.9 ± 32.8 A	43.3 ± 22.9 A	39.9 ± 25.7 A	0.221
ALP	66.9 ± 34.1 A	86.8 ± 33.5 A	146.1 ± 103.9 B	<0.01
INR	1.10 ± 0.09 B	1.4 ± 0.26 B	1.17 ± 0.28 A	<0.01
Bilirubin (mg/dl)	0.77 ± 0.27 B	2.15 ± 2.26 A	1.40 ± 1.35 AB	<0.01
AFP (ng/ml)	3.9 ± 4.3 A	6.9 ± 4.3 A	204.6 ± 15.7 B	<0.01
GPC3	0.4 ± 0.83	0.8 ± 0.85	1.1 ± 1.13	0.022
PEG 10	2.9 ± 5.8	1.14 ± 2.3	4.4 ± 5.5	0.01
MDK	0.7 ± 1.7	0.8 ± 1.7	2.26 ± 4.3	<0.01
SERPINI 1	0.74 ± 2.12	1.03 ± 2.4	1.64 ± 2.69	<0.01
QP-C	1.8 ± 2.12	0.35 ± 0.7	0.75 ± 2.78	0.025

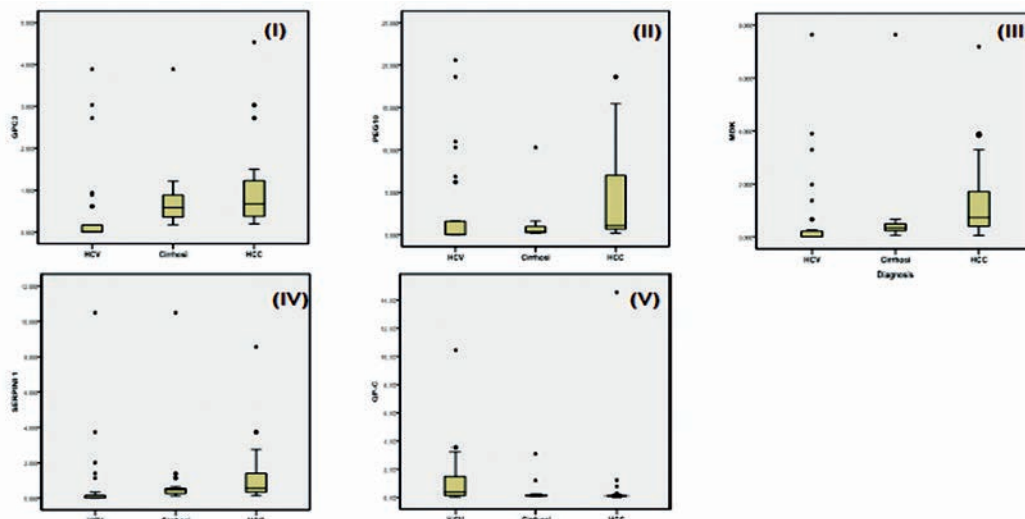
Means with different letters (A, B, C) within the same row are significantly different (p ≤ 0.05)

mL as (31%, 52% and 17%), respectively (Table II). In relation to different tumor-related variables, the genetic markers tested in HCC patients showed no significant correlation between any of the studied markers and the number of tumor nodules, the size of the lesions, extra hepatic spread or the AFP level (Table III). Moreover, no correlations were found between these gene markers and the patients' age, Child Pugh score and the different laboratory parameters except for the serum bilirubin which showed statistically significant negative correlation with all the gene markers.

A receiver operating characteristic (ROC) curve was constructed for the studied markers to estimate the best cutoff which could improve the specificity and sensitivity of these markers to differentiate between the studied groups. ROC

curve for HCC group versus non-HCC group showed that at a cutoff value of 0.18, GPC-3 showed 100% sensitivity, 48% specificity, and the area under the curve 0.737 (p= 0.001). At a cutoff value of 0.30, MDK showed 89% sensitivity and 64% specificity. The area under the curve was 0.79 (p = 0.000). At a cutoff value of 0.34, PEG-10 showed 82% sensitivity, 58% specificity and the area under the curve 0.74 (p=0.00). At a cutoff value of 0.13, SERPINI1 showed 100% sensitivity and 47% specificity .The area under the curve was 0.74 (p=0.00) (Table IV, Fig. 2).

Looking for a better diagnostic role of the candidate gene expression in the HCC group, simultaneous measurement of all genetic markers together demonstrated that the sensitivity was 100%, specificity 33.3%, PPV 48.1% and NPV 100%.



**Fig. 1.** Box plots showing the level of gene expressions in the studied groups: (I) GPC3, (II) PEG10, (III) MDK, (IV) SERPIN1, (V) QP-C.

**Table II.** Tumor characteristics and AFP stratification in HCC group

Variant	Number of patients N:29	Percentage
Tumor size (cm)		
<3cm	5	17.2%
3-5cm	15	51.7%
>5cm	9	31%
No of nodules		
(1-2 nodules)	20	69%
> 2 nodules	9	31%
Metastases (nodal or blood)	4	13.8%
PV invasion	1	3.4%
AFP (ng/ml)		
<10	9	31%
10-200	15	52%
>200	5	17%

**Table III.** Genetic markers in the HCC group in relation to tumor-related variables

Tumor-related variables	GPC-3	PEG10	MDK	SERPINI 1
AFP (ng/ml)				
Normal (<10)	0.53 A	1.87 A	0.73 A	0.97 A
10- 200	0.92 A	3.60 A	0.55 A	0.58 A
>200	0.42 A	0.90 A	1.64 A	0.55 A
P value	0.067	0.480	0.335	0.856
Number of tumor nodules				
Diffused (>2 nodules)	0.559	1.42	0.8545	0.6555
Undiffused (1-2 nodules)	0.871	1.13	0.743	0.607
P value	0.117	0.501	0.737	0.867
Tumor size				
<3 cm	0.855	0.72	0.631	0.6895
3-5 cm	0.8405	1.075	1.0925	0.5775
>5 cm	0.6745	3.34	0.8545	0.6635
P value	0.851	0.813	0.686	0.672
Extra hepatic spread				
No metastases	0.751	1.13	0.7785	0.599
Nodal metastases	0.759	2.81	2.1885	1.668
P value	0.844	0.950	0.325	0.237

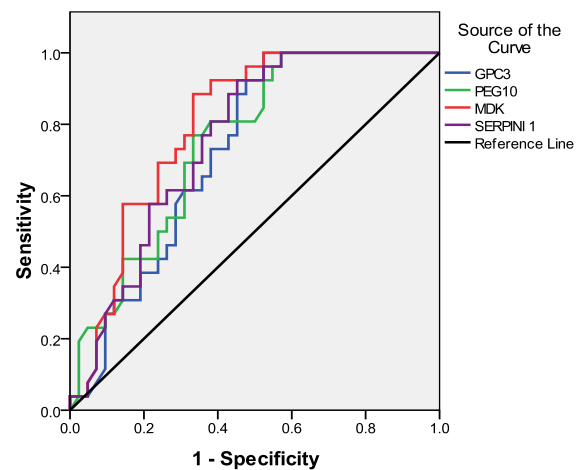
A in the same row: significantly different

In addition, a simultaneous measurement of AFP with the gene markers in HCC versus non HCC group showed little improvement of the sensitivity at the expense of the specificity (Table V).

**Table IV.** Diagnostic performance of markers for the discrimination of patients with HCC from non HCC patients

	AUC	p value	95% Confidence Interval		Best cutoff	Sensitivity	Specificity	PPV	NPV
			Upper	Lower					
GPC-3	0.737	0.001	0.852	0.622	0.184	1.00	0.48	55.1%	100.0%
MDK	0.799	0.000	0.900	0.698	0.302	0.89	0.64	60.0%	90.6%
PEG-10	0.744	0.000	0.855	0.633	0.345	0.82	0.58	54.8%	83.9%
SERPINI1	0.764	0.000	0.871	0.657	0.131	1.00	0.47	52.9%	100.0%

PPV: positive predictive value; NPV: negative predictive value

**ROC Curve****Fig. 2.** ROC Curve analysis showing the diagnostic performance of GPC3, MDK, PEG10, SERPINI1 expression in blood for discriminating patients with HCC from non HCC patients.

## DISCUSSION

Primary tumors rarely have deadly consequences, while metastatic disease accounts for around 90% of the mortality due to solid tumors [14]. Therefore, the development of new sensitive methods that allow the detection of cancer dissemination, most notably in the common carcinomas, before full blown clinically detectable gross metastatic deposits are established, is of tremendous utility to help physicians in treatment decisions.

Localized and metastatic cancers give rise to circulating tumor cells (CTCs) which are detectable in the blood stream. Several studies have highlighted the prognostic significance of the presence and number of CTCs, particularly in patients with metastatic disease. CTCs can be easily obtained from peripheral blood for which frequent sampling is usually accepted by patients and their treating physicians.

Nucleic acid-based methods for the characterization of CTCs are considered to be more sensitive than cytometry and immunocytochemistry methods [15, 16], achieving specificity through oligonucleotide primers designed for detection of genes of interest. Thus, molecular characterization of CTCs may also be useful for the assessment of predictive bio-markers in real-time and for the development of tailored therapies.

Different techniques have been described to separate CTC; in the present study the standard Ficoll density gradient centrifugation system is used for improved tumor cell enrichment in the blood of studied patients because of its

**Table V.** Simultaneous measurement of AFP and the new markers

	Sensitivity	Specificity	PPV	NPV
All markers	100%	33.3%	48.1%	100%
AFP and GPC3	100%	42.9%	52.9%	100%
AFP and MDK	100%	55.6%	57.4%	100%
AFP and PEG10	96.4%	51.1%	55.1%	95.8%
AFP and SERPINI1	100%	42.2%	51%	100%

PPV: positive predictive value; NPV: negative predictive value

simplicity, low cost and reasonable recovery rate of CTC as previously discussed [17].

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in some areas of the world. In most cases, HCC is diagnosed at a late stage and its prognosis is generally poor [18]. Therefore, the aim of the present study was to investigate and determine the expression profile of five HCC linked candidate genes: GPC3, PEG10, MDK, SERPINI1 and QP-C in the peripheral blood of Egyptian patients with HCV related HCC. The used assays identify genes known to be associated with HCC by using primers and probes designed to recognize these genes. This method boasts a high analytical sensitivity.

In the current work, no significant gender difference was discovered among studied patients but most of them were males. This can be explained by the difference in exposure to risk factors which is higher in males than females; a viral infection, alcoholism and smoking [19, 20]. In addition, it has been speculated that estrogen and androgens could modulate hepatocarcinogenesis [21]. As regards the age, there was no significant difference between the cirrhosis and HCC groups. However, it was significantly decreased in the CHC group. This age difference is compatible with the age range of Egyptian cases of HCC as reported. The incidence of 64.9% of HCC cases in Egypt is between the age of 50 and 69 years [22]. That may be explained by the fact that liver cancer is generally attributed to HCV and chronic HCV most probably leads to carcinogenesis after 10-30 years following infection or may be due to late diagnosis in Egypt [23]. The patients in the liver cirrhosis group were evenly distributed in the three classes of the Child Pugh score. Most of the patients in the HCC group were Child A, presented at an early stage without manifestations of advanced disease thanks to screening programs. Also, most of the patients in our study had an AFP level below 200ng/ml, but there is a statistically significant difference between the HCC and non HCC groups that can be explained by its production by malignant cells and even in the fibrotic tissue. Alpha-fetoprotein has many diagnostic problems; it may be higher in HCV related cirrhosis [24]. In a large multicenter study, researchers verified that AFP had a poor sensitivity in the diagnosis of HCC and that its prognostic value is limited. AASLD recommends that AFP alone should no longer be used to screen at risk patients for HCC [25].

In our study, 68.9% of the patients in the HCC group had lesions below 5cm in diameter with only one single or two

nodules. Identification of the discussed markers in this group of patients helped in the diagnosis of HCC at a relatively early stage of the disease.

Glypicans are proteoglycans that interact with growth factors and modulate their activities; hence, they play an important role in cell growth, differentiation and migration [26, 27]. Glypican 3 (GPC3) was suggested as a possible tumor marker for HCC, since the levels of GPC3 were significantly high in the serum of HCC patients, but undetectable in healthy donors and patients with benign liver diseases [28]. Moreover, it was found to be expressed in small tumors, indicating its potential as a diagnostic marker for early stage HCC [29]. In our study, the GPC3 mRNA level in the blood was significantly higher in the HCC group as compared to liver cirrhosis and CHC groups. Although our results are contradictory to Wang et al [30] who did not detect any significant difference in GPC3 mRNA expression in the PB between the HCC and healthy volunteers, patients with chronic viral hepatitis and cirrhosis, the results are similar to Kandil and Cooper [31] who revealed that GPC3 was expressed in 70-80% of their studied HCC lesions while being virtually absent in the normal liver. Yan et al [32] detected GPC3 mRNA in the peripheral blood of 76% of HCC patients while it was not detected in samples of healthy subjects, patients with hepatitis B, cirrhosis, hepatic hemangioma, or hepatic metastasis. In another Egyptian study, GPC3 mRNA was detected in 100% of HCC cases, 5% of liver cirrhosis and none in normal control subjects [33]. This variability can be attributed to *in vitro* instability of mRNA, differences concerning laboratory techniques, primer selection, time between sample collection and processing, and different patient populations.

In 1988, Kadomatsu et al [34] first isolated a Midkine (MK) cDNA clone by differential hybridization and reported that MK was intensely expressed in early differentiation stages of embryonal carcinoma cells. This was followed by other studies that reported that MK can exert cancer-related activities in the process of carcinogenesis, including transformation, fibrinolysis, cell migration, cell survival, anti-apoptosis, and angiogenesis [35-37]. For PEG10, it was first described by Ono et al [38] as an imprinted gene with an active paternal allele but silent maternal allele and is generally not expressed in the normal adult liver. The PEG10 gene is activated in a variety of human cancers including leukemia, breast cancer, prostate cancer, and pancreatic cancer [39]. A functional role for PEG10 in the growth-promoting activities has also been demonstrated in HCC cells [40]. In our study, we showed that PEG10 expression was significantly higher in HCC patients compared to non HCC patients, a similar over expression of MK level was found among our HCC group. Comparative results were previously reported by Tsou et al [40] who identified PEG10 as a novel gene having an elevated level of expression in the majority of the HCC samples and Aridome et al [41] who showed that the MK mRNA level was higher in HCC specimens than in the corresponding non-cancerous tissues. Kato et al [42] also reported increased MK expression in HCC at the mRNA and protein level. Before our present study, there were no reports, to our knowledge regarding the MK mRNA expression in the peripheral blood of patients with HCC.

Serpins are Serine Protease Inhibitors targeting many proteases [43–48]. Through their ability to reduce proteolysis, serpins were predicted to impair extracellular matrix degradation and consequently cancer cell invasion and metastasis. However, serpinE1 (or plasminogen activator inhibitor-1, PAI-1) has been reported to promote angiogenesis and to induce tumor cell migration [49, 50]. It was found that an increased expression of SERPINI1 is associated with most HCC samples, including those with normal serum AFP and small tumor size [9]. SERPINI1 expression in peripheral blood of our HCC patients was significantly higher than other groups. This was also demonstrated by Spano et al [51] who revealed a molecular signature of 11 genes upregulated in HCC, SERPINI1 being one of them. The up-regulation of SERPINI1 gene is in agreement with its location on chromosomal region gained in HCC.

Concerning the ubiquinone-binding protein (QP-C), it is a nuclear-encoded component of ubiquinol-cytochrome c oxidoreductase in the mitochondrial respiratory chain and plays an important role in the electron transfer as a ubiquinone-QP-C complex [52]. It was found that QP-C was over expressed in HCC samples [9] while in our study, this gene did not show significant expression in the HCC group. This finding can be explained by the different types of patients as most of the patients in their study were post HBV HCC in contrast to our patients who were post HCV HCC, and in addition the use of PBMC for the study of gene expression instead of liver tissue.

In our study, no significant correlation was found between the gene expression level and the tumor size. This was previously proved by Jia et al [9] who revealed significant expression of those markers in most of HCC samples including small sized tumors.

There was no significant correlation between any of the studied markers and AFP levels in contrast to the study of Jia et al [9], which showed that expression levels of MK and SERPINI1 were significantly higher in AFP-high samples than that of AFP-normal samples. The expression levels of GPC3 and PEG10 were also higher in AFP-positive than that of the AFP-normal samples, although the differences were statistically insignificant.

However, simultaneous measurement of all the studied markers together led to increased sensitivity (100%) as against the specificity with PPV of 48% and NPV of 100%. This differs from Jia et al [9] who reported a high sensitivity and specificity for the five-gene signature to classify new HCC samples. The positive predictive value and negative predictive value were 100% and 80%, respectively. However, their study measured the five genes mRNA in HCC tissue not in the peripheral blood, which might indicate that the locally expressed markers may be a more sensitive predictor of HCC in tissue than in peripheral blood. However, blood samples are much more easily obtained for monitoring HCC vulnerable patients than tissue biopsies.

## CONCLUSION

Our results demonstrated that GPC3, PEG10, MDK and SERPINI1 mRNA levels in peripheral blood were significantly higher in HCC patients as compared to chronic HCV hepatitis and liver cirrhosis groups, while QP-C mRNA was higher in patients with chronic HCV hepatitis. Gene expression levels

were independent of the tumor size, number of tumor nodules, extrahepatic metastases or AFP level.

**Conflicts of interest:** None to declare.

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