Evaluation of Transforming Growth Factor- β $_1$ Gene Expression and Polymorphism in Diagnosis of Hepatocellular Carcinoma in HCV-Infected Patients

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Abstract:

Background: Transforming growth factor-beta1 (TGF-β1), a member of transforming growth factor beta family, functions as a multi-functional cytokine and plays a key role in cellular growth, proliferation and differentiation. **Aim of the work**: To evaluate the role of TGF-β1 polymorphisms 509 C>T and TGF-β1 Gene expression in diagnosis of hepatocellular carcinoma (HCC) in cirrhotic Egyptian patients due to hepatitis C virus (HCV). **Subjects and methods**: Thirty five cirrhotic with HCC patients, thirty cirrhotic without HCC patients and 20 healthy volunteers were enrolled in this study. TGF-β1 gene expression in peripheral blood was quantitated using real-time polymerase chain reaction and molecular study of TGF-β1 509 C>T (rs1800469) polymorphism by Taqman allele discrimination was done for all subjects. **Results:** Showed that TGF-β1 -509 CT genotype was the most frequent genotype in HCC patients and the most frequent allele was T allele in HCC patients, without significant difference of TGF-β1 -509 polymorphism in the studied groups. TGF-β1 gene expression was significantly higher in HCC patients (mean 3.19±1.8) than in cirrhotic without HCC patients (mean 1.76±0.95) ($p < 0.001$) and normal controls (mean 1.00 ±0.8) ($p < 0.001$), and AFP $\geq$ 41 (ng/ml) and TGF-β1 gene expression $\geq$ 1.85 are diagnostic for HCC presence. **Conclusion:** TGF-β1 gene expression in the peripheral blood may be used as a molecular marker for HCC diagnosis, and TGF-β1 polymorphism 509 C>T was not associated with the risk of HCC in HCV-cirrhotic patients.

Key words: Transforming growth factor-beta 1 (TGF-β1), Hepatocellular carcinoma (HCC), Hepatitis C virus (HCV).

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer [1]. HCC usually develops in patients with liver cirrhosis [2], and represents the second most common cause of cancer-related deaths [3]. Most of the HCC cases develop in the presence of cirrhosis related to viral hepatitis. In particular, hepatitis C virus (HCV) and hepatitis B virus (HBV) infections are considered major risk factors for HCC worldwide. However, recent studies reported increasing number of HCC in nonalcoholic fatty liver disease (NAFLD) [4].

In Egypt, it is believed now that HCC is one of the common malignancies and a leading cause of death due to high prevalence of cirrhosis related to chronic HCV. Previous years showed that, there is an increase in its incidence and it is expected that the number of cases continues to grow [5].

Diagnosis of HCC in cirrhotic patients should be based on non-invasive criteria and/or pathology. Non-invasive criteria can only be applied to cirrhotic patients for nodule(s) ≥1 cm, based on imaging techniques obtained by multiphasic CT, dynamic contrast-enhanced MRI or contrast-enhanced US (CEUS). Diagnosis is based on the identification of the typical hallmarks of HCC, which differ according to imaging techniques or contrast agents [6].

Detailed analysis and characterization of molecular, genetic and epi-genetic events would revolutionize early diagnosis of HCC [7]. Gene and protein expression profiling will allow better screening of different stages of HCC as well as establishment of criteria for targeted therapies [8].

Transforming growth factor-β1 (TGF-β1) is a well-known developmental factor involved in regulation of cell proliferation, differentiation, invasion and inflammation. In mammals, the TGF-β family regulates many cellular functions playing an important role in cell growth, differentiation, apoptosis, extracellular matrix (ECM) production, immunization and even embryonic development [9]. TGF-β1 plays an important role in the pathogenesis of various liver diseases, such as fibrosis and cirrhosis [10].

Genetic testing of TGF-β1 -509 C/T polymorphism may be useful for identifying high-risk individuals such as subjects with HCV infection, and the results may encourage the higher risk population to receive medical examinations frequently for early detection of HCC [11].

This study aimed to evaluate TGF-β1 gene expression in peripheral blood and its polymorphism −509C/T in diagnosis of HCC in chronically infected Egyptian HCV patients.

Subject and methods

This case – control, cross – sectional study was conducted on 65 patients and 20 healthy volunteers, admitted to the Hepatology, Gastroenterology and Infectious Diseases Department in Benha University Hospital in period from February 2018 to October 2018 in cooperation with the Medical Biochemistry and Molecular Biology

Department. The protocol of this study was approved by the Ethical Committee of the Faculty of Medicine, Benha University and informed consent was taken from each subject before participation in this study.

The subjects were divided as follows:

- **Group (I):** Included 20 apparently healthy subjects served as a control group.
- **Group (II):** Included 30 cirrhotic patients due to chronic HCV infection without HCC.
- **Group (III):** Included 35 cirrhotic patients due to chronic HCV infection with HCC.

❖ Patients < 18 years old, patients with liver cirrhosis (LC) due to causes other than HCV (as HBV infection, autoimmune and metabolic liver diseases), patients with

❖ other liver malignancies (as haemangioma, adenoma and hepatoblastoma), patients with metastatic liver cancer, patients with portal vein invasion, patients received prior therapy for HCC lesion, patients with recurrent HCC, patients with past history or on antiviral therapy were excluded from this study.

❖ Patients with HCC was diagnosed by serum α-fetoprotein elevation ≥ 200 ng/dl, abdominal ultrasound and triphasic CT.

❖ All cases and controls were subjected to full history taking and thorough clinical examination and laboratory investigations.

Sample collection:

Peripheral venous blood sample (6 ml) was obtained from each subject under complete aseptic conditions. The blood sample will be divided into 3 parts: the first part (1ml) was put into sterile vacutainer EDTA tube; 0.5 ml for CBC, and 0.5 ml for genotyping. Blood for TGF- β_1 509 C>T genotyping was stored at –80 ^{o}C in nuclease-free sterile eppendorff tubes. The second part (0.9 ml) was withdrawn into a tube containing tri-sodium citrate (concentration 3.8%)

solution in a ratio of 9:1 for determination of PT concentration, activity and INR. The third part (~ 4 ml) was left to clot and serum was separated for determination of TGF-β_1 gene expression, other serological and biochemical investigations.

- **Laboratory investigations were done as follow:**

a. Complete blood picture (CBC) performed by automated hematology analyzer Sysmex XS-1000i **(Sysmex, Japan)**[12].

b. ESR (ml/hour) [13].

c. Random blood glucose (mg/dl) [14].

d. Kidney function tests: serum creatinine (mg/dl) and blood urea (mg/dl) [15].

e. Liver profile tests including:

- Serum alanine transeferase (ALT) and aspartate transeferase (AST) (U/dl) [16].
- Serum albumin (g/dl) [17].
- Serum bilirubin (total and direct) (mg/dl) [18].

f. Prothrombin time (PT) (sec), concentration (PC) (%) and international normalized ratio (INR) using Behring Fibrin timer II from (Behring, Germany) [19].

g. Viral markers: HCV Abs [20] and HBsAg [21] by third generation of enzyme linked immuno-sorbant assay (ELISA)

***h.* Serum alpha feto-protein level (AFP)** (ng/ml) [22] by ELISA.

▪ Viral markers and AFP were performed by ***Tecan Infinite spectrophotometer 50 ELISA Reader (Singapore).*** The other tests were done by ***Microtech spectrophotometer (Vital Scientific, Netherlands).***

i. Serum TGF- β_1 gene expression by real-time polymerase chain reaction (RT-PCR) as follow:

1. Total mRNA Extraction:

It was performed using 200 µl serum of each subject via Plasma/Serum RNA Purification Kits (Norgen Biotek Corporation, Thorold, ON, Canada), including digestion with DNase I according to the manufacturer instructions.

2. Quantitation of extracted RNA:

Ultraviolet spectrophotometric quantification of RNA by nanodrop 2000 Spectrophotometer *(Thermo Fisher Scientific, Wilmington, USA),*was used. Pure RNA preparations have optical density (OD) ratio at 260/280 nm of 1.9-2.3 [23].

3. Relative quantitation (RQ) of mRNA of the respective gene by RT-

PCR using Syber green reagents in 2 steps:

The first step RT-PCR was for conversion of RNA into complementary DNA (cDNA) in a Veriti™ Thermal Cycler (Applied Biosystems), using Maxime RT PreMix Kit (Intron Biotechnology). The concentrations of components in the RT mixture were: 0.25 ug for total RNA, 0.1 ug for poly (A) RNA and the reaction was completed up to 20 µl with distilled water. The thermal cycling conditions were: 45 ^{o}C for 60 min. (cDNA synthesis) and 95 ^{o}C for 5 min. (RTase inactivation).

The second step RT-PCR was for quantitation of TGF-1β gene expression of mRNA in a ***Stepone*** real time PCR system ***(Applied Biosystem, Singapore)***. Singleplex reactions were done. This step was performed using ***SensiFAST^{TM} Sybr Hi-Rox Kit (Bioline Reagents Ltd, United Kingdom).*** Human GAPDH was the endogenous housekeeping gene. Melting curve analysis was done in each run to confirm specificity of real-time PCR assay. The primers were as follow: **TGF-β1; FP:** 5'-TCTGTGGGGATGTGACA GGA-3' and RP: 5'-TTGGTTGTA GAGGGCAAGGAC-3' [24] and **GAPDH; FP:** 5'-AAGGGCCCTGA CAACTCTTT-3' and RP: 5'-CTCCC CTCTTCAAG GGGTCT-3' [24].

The components of singleplex PCR were: 10 µl SensiFAST SYBR Hi-ROX Mix (2X), 0.8 µl FP, 0.8 µl RP, 2 µl cDNA

and 6.4 µl Nuclease free water. The thermal cycling conditions were: initial denaturation (95^{o}C/5min), 40 cycles (denaturation; 95^{o}C/15sec., extension; annealing; 55^{o}C/1min and 72^{o}C/20sec.) and then melting curve analysis was applied to ensure primer specificity.

According to the RQ manager program, the data were produced as sigmoid shaped amplification plots in which the number of cycle was plotted against fluorescence (when using linear scale). The samples of control group were used as calibrators so the expression levels were set to 1. The relative quantities of human TGF-1β mRNA were normalized against the relative quantities of the endogenous control (human GAPDH) so gene fold expression changes were calculated using the equation $2^{-\Delta\Delta CT}$ [25].

j- Molecular study of TGF- β_1 509 C>T (rs1800469) polymorphism: It was performed by Taqman allele discrimination as follow:

(A) Genomic DNA extraction:

DNA was extracted from 200µl blood sample; using ***Purelink® Genomic DNA minikit Catalog No. K1820-01***

(Invitrogen, Life Technologies) according to the manufacturer's instructions. RNase A digestion was performed to degrade RNA in the sample also digestion by proteinase K was done for efficient lysis of cells. Elution of DNA was done by 50 elution buffer. Ultraviolet spectrophotometric quantification of double stranded genomic DNA by nanodrop 2000 spectrophotometer ***(Thermo Fisher Scientific, Wilmington, USA)*** was done. Readings were taken at wave lengths 260 and 280 nm. according to that reported by [23].

Pure preparations of DNA have OD260/OD280 of 1.7 - 2.0. The extracted DNA was kept at -20^{o}C till the real-time PCR was performed.

(B) Real-time PCR for detection of TGF-1β (rs1800469):

It was done by 5' Nuclease Taqman SNP Genotyping Assay Technology. In 20 µl reaction, genomic PCR amplification was done using Taqman 5´ allele discrimination assay *(Applied Biosystem, Foster City, California, USA).* TGF-1β rs1800469 assay contained sequence specific primers for both alleles (C and T) and 2 Taqman probes; one probe labeled with VIC dye detects the C allele and the other labeled with FAM detects the T allele. The assay supplied was 40X and was diluted before real time PCR mix was done to 20X working stock with 1X TE buffer.

Components of PCR amplification mixture were: 10 µl Taqman Universal PCR master mix, No AmpErase UNG (2X), 1 µl 20X Working Taqman SNP assay, 20 ng DNA template and up to 20 µl nuclease-free water. Amplification was done in Stepone Real-Time PCR System *(Applied Biosystem, Foster City, USA).* The following thermal cycling conditions were run: Pre-PCR Read (60^{o}C for 30 sec.), Amplitaq Gold Enzyme activation (95^{o}C for 10 min.) and 40 cycles (denaturation; 92^{o}C for 15 sec. and anneal/extend; 60^{o}C) and Post-PCR Read (60 ^{o}C for 30 sec.). Two no template controls (NTCs) using DNase free water were essentially done in each run. The success rate for this genotyping was 100%.

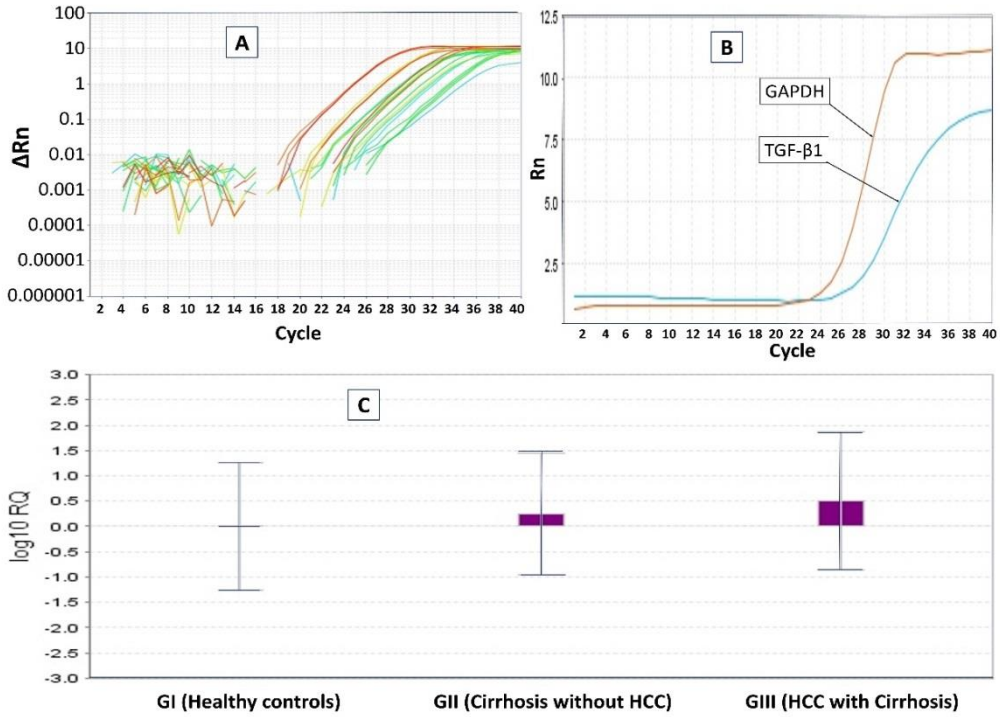

Figure (1): TGF-β1 gene expression: A; amplification plot of TGF-β1 and GAPDH (endogenous control) in the studied groups, B: amplification plot of TGF-β and GAPDH in a single sample, C: Gene expression plot of TGF-β1 in the studied groups.

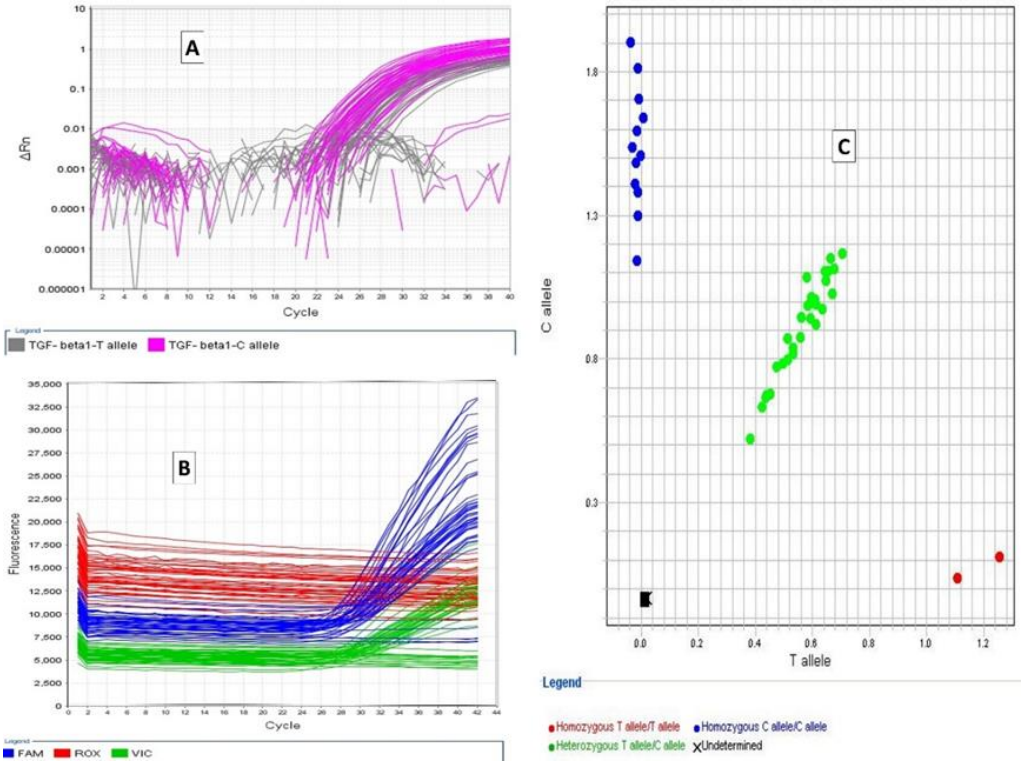

Figure (2): TGF-β1 (rs1800469); A: amplification plot, B: multicomponent plot and C: allele discrimination plot.

Statistical analysis:

The SPSS 12.0 statistical software was used for statistical analysis (Spss Inc, Chicago, ILL Company). Categorical data were presented as number and percentages while quantitative data were expressed as mean ± standard deviation and range. Chi square test (X^2) were used to analyze categorical variables, odds ratios (OR) were calculated when applicable.

Quantitative data were tested for normality using Shapiro-Wilks test, assuming normality at P>0.05. Difference among 3 independent means was analyzed using analysis of variance (ANOVA) for parametric variables or Kruskal Wallis test (KW) for non-parametric ones. ROC curve was used to determine cutoff value of the studied markers with optimum sensitivity and specificity in early diagnosis of HCC. Uni and multi variable logistic regression analysis were run to detect the significant predictors of HCC. The accepted level of significance in this work was stated at 0.05 (P <0.05 was considered significant). Genotype distributions in the studied groups were in Hardy-Weinberg equilibrium for gene polymorphisms

Results

This study conducted on 85 subjects attending Department of Hepatology, Gastroentrology and Infectious Diseases in Benha University Hospital, in the period from February 2018 to October 2018. A total of 65 Egyptian subjects with chronic HCV (30 cirrhotic without HCC + 35 cirrhotic with HCC) and 20 healthy volunteers were enrolled in our study. Age and sex of cases and healthy volunteers are summarized in **(Table 1).**

No significant differences were found in age and gender distributions between the cases and controls. Platelets (PLTs), serum creatinine, ESR, AST, total bilirubin, serum albumin, INR and AFP levels were significantly different among the studied groups **(Table 2).** However, there was no statistically significant difference between the studied groups as regard Child-Pugh classification as shown in **(Figure 3).**

Regarding TG-β1 gene expression **(Table 3),** there was highly statistical significant difference between controls and cirrhotic patients with HCC and between cirrhotic patients with and without HCC (p<0.001 for all).

As regard, TGF-β1 509 C > T genotype and allele frequencies **(Table 4),** there was no statistical significant difference between cirrhotic without HCC and cirrhotic with HCC groups in comparison

with the control group. The highest gene expression was with TT genotype patients **(Table 5).**

The current study found that, AFP and gene expression can significantly predict HCC at the shown cut off values (≥ 41ng/ml and ≥ 1.85 respectively). Gene expression is more sensitive (88.6%) but AFP is more specific (84%) **(Table 6).**

Regarding univariable binary logistic regression analysis revealed that, age > 58 years, creatinine level > 1.3 (mg/dl), serum albumin level < 2.5 (g/dl), ESR > 80, AFP ≥ 41(ng/ml) and level of gene expression ≥ 1.85 fold increase were significant risk factors for HCC. Multivariable binary logistic regression analysis showed that AFP ≥ 41 (ng/ml) and level of gene expression ≥ 1.85 fold increase were significant independent predictors of HCC **(Table 7).**

Table (1): General and clinical characteristics of the studied groups

Variables		Group I (Control) (n=20)		Group II (Cirrhotic without HCC) (n=30)		Group III (Cirrhotic with HCC) (n=35)		Test &P	P of multiple comparisons
Age (years)	**Mean±SD**	57.2±8.9		57.5±9.0		62.0±8.7		2.79* (0.067) NS	P_1=1.0 P_2=0.16 P_3=0.13
	Range	45-73		40-80		45-80			
		No.	**%**	**No.**	**%**	**No.**	**%**	χ^2	
Sex	**Male**	9	45.0	11	36.7	20	57.1	0.34	P_1=0.55
	Female	11	55.0	19	63.3	15	42.9	0.75 2.71	P_2=0.38 P_3=0.099

P1: between group I and II, P2: between group I and III, P3: between group II and III *ANOVA

Table (2): Comparison between the studied groups as regard laboratory findings

Variables	Group I (control) (n=20)		Group II (cirrhotic without HCC) (n=30)		Group III (cirrhotic with HCC) (n=35)		Test & P	P of multiple comparisons
	Mean	±SD	Mean	±SD	Mean	±SD		
PLTs (c/µl)	272.7	79.1	114.7	76.5	132.1	92.9	24.1 &<0.001 (HS)*	P_1<0.001 (HS) P_2<0.001 (HS) P_3<0.001 (HS)
S.creatinine (mg/dl)	0.89	0.21	1.10	0.75	1.41	0.79	15.7 &<0.001 (HS)**	P_1=0.01 (S) P_2=0.001 (HS) P_3= 0.024 (S)
ESR (ml/hour)	13.5	7.96	53.0	36.2	81.0	38.4	41.2& <0.001(HS)**	P_1<0.001 (HS) P_2<0.001 (HS) P_3<0.001 (HS)
AST (U/dl)	32.5	12.3	46.6	21.6	54.5	39.9	11.07 &0.004 (S) **	P_1=0.29 P_2=0.027 (S) P_3= 0.85
T. bilirubin (mg/dl)	0.99	0.23	3.6	3.42	3.2	4.54	30.2 & <0.001 (HS) **	P_1=0.009 (S) P_2=0.03 (S) P_3=1.0
S. albumin (g/dl)	4.22	.48	2.68	.64	2.63	.55	56.3* &<0.001 (HS)*	P_1<0.001 (HS) P_2<0.001 (HS) P_3<0.001 (HS)
INR	1.03	0.09	1.43	0.35	1.85	2.31	31.2&<0.001 (HS) **	P_1=0.012 (S) P_2=0.003 (S) P_3=0.023 (S)
AFP (ng/ml)	1.74	1.48	33.8	32.44	238.7	232.19	56.3 & <0.001 (HS) **	P1<0.001 (HS) P2<0.001 (HS) P3<0.001 (HS)

P1: between group I and II, P2: between group I and III, P3: between group II and III

*: ANOVA, ** : KW test , SD: standard deviation, PLTs : platelets, S.: serum, ESR : Erythrocyte sedimentation rate, AST: Aspartate transeferase, T.: total, INR : International normalized ratio, AFP : Alpha-fetoprotein.

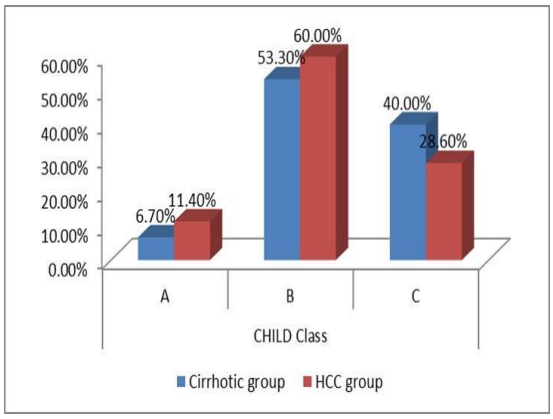

Figure (3): Bar chart showing Child-Pugh classifications among studied patients (cirrhotic with and without HCC)

Table (3): Comparison between the studied groups regarding TGF-β_1 gene expression

Variables	Group I (control) (n=20)		Group II (cirrhotic without HCC) (n=30)		Group III (cirrhotic with HCC) (n=35)		ANOVA & P	P of multiple comparisons
	Mean	±SD	Mean	±SD	Mean	±SD		
TGF-β1 Gene expression (fold change)	1.00	0.80	1.76	0.95	3.19	1.8	17.6 & <0.001 (HS)	P_1=0.19 P_2<0.001 (HS) P_3<0.001 (HS)

P1: between group I and II, P2: between group I and III, P3: between group II and III SD: standard deviation

Table (4): Comparison between the studied groups as regard TGF-β1 509 C > T genotype and allele frequencies

Variables	TGF-β1 509 C > T	Controls (n=20)		Group II (cirrhotic without HCC) (n=30)		OR (95%CI)	P	Controls (n=20)		Group III (cirrhotic with HCC) (n=35)		OR (95%CI)	P
		No.	%	No.	%			No.	%	No.	%		
Genotypes	CC	9	45.0	11	36.7	**Ref.**		9	45.0	7	20.0	**Ref.**	
	CT	9	45.0	16	53.3	1.45 (0.4-4.8)	0.54	9	45.0	20	57.1	2.85 (0.8-10.1)	0.1
	TT	2	10.0	3	10.0	1.22 (0.16-9.0)	0.84	2	10.0	8	22.9	5.1 (0.8-32.3)	0.08
Allele	C	27	67.5	38	63.3	1.2 (0.5-2.8)	0.66	27	67.5	34	48.6	2.2 (0.97-4.9)	0.057
	T	13	32.5	22	36.7			13	32.5	36	51.4		

OR: odd ratio, CI: confidence interval

Table (5): Gene expression according to genotype in HCC group

Variables	CC (n=7)		CT (n=20)		TT (n=8)		ANOVA & P	P of multiple comparisons
	Mean	±SD	Mean	±SD	Mean	±SD		
TGF-β 1Gene expression (fold change)	1.93	0.79	2.55	0.57	5.87	2.21	27.6 &<0.001 (HS)	CC ≠ CT=0.72 CC ≠ TT<0.001 CT ≠ TT<0.001

SD: standard deviation, ≠ : versus.

Table (6): ROC curve analysis for the performance of AFP, serum TGF-β1 and gene expression in the prediction of HCC

Variables	Cut off	Sens%	Spec%	PPV%	NPV%	AUC	95%CI	P
AFP (ng/ml)	≥ 41	82.9 %	84 %	78.4 %	87.5 %	0.903	0.83-0.97	<0.001 (HS)
Gene expression (fold change)	≥ 1.85	88.6 %	74 %	70.5 %	90.2 %	0.853	0.77-0.93	<0.001 (HS)

ROC: receiver operating characteristic, PPV: positive predictive value, NPV: negative predictive value, AUC: area under ROC curve, CI: confidence interval.

Table (7): Multivariable binary logistic regression analysis for the predictors of HCC

Variables	Multivariable logistic regression			
	β	Adjusted OR	95%CI	P
Age >58 (years)	13.5	6.1	0.23-35.7	0.91
Creatinine > 1.3 (mg/dl)	10.9	3.8	0.21-24.6	0.96
Serum albumin< 2.5 (g/dl)	42.1	10.1	0.97-35.8	0.70
ESR > 80 (ml/hour)	83.6	16.4	0.77-44.9	0.67
AFP ≥ 41 (ng/ml)	157.6	29.7	7.5-68.3	0.004 (S)
TGF-β1 Gene expression (fold change) ≥ 1.85	149.0	25.7	5.8-71.5	0.009 (S)
Constant		-158.5		

OR: odd ratio, CI: confidence interval, ESR: Erythrocyte sedimentation rate, AFP: Alpha-feto protein

Discussion

Hepatocellular carcinoma (HCC) is considered to be the most common primary cancer of the liver [26]. It accounts for 75–85% of primary liver cancers and is the second leading cause of cancer death in East Asia and sub-Saharan Africa and the sixth most common in Western countries [26, 27].

Egypt has a high incidence of HCC about 21% in cirrhotic Egyptian patients. HCV and HBV infections, diabetes and smoking are the main determinants of HCC development in Egypt. There is a synergistic effect of many risk factors. An active surveillance and secondary prevention programs for patients with chronic hepatitis are the most important steps to reduce the risk of HCC [28].

Egypt has the highest prevalence of HCV in the world [29]. HCV infection and its complications are among the leading public health challenges in Egypt

with 13.8% of population infected [30], and in these patients, the risk of HCC is increased 17-fold [31].

Several studies that use molecular signatures have provided promising strategy for the prediction of HCC prognosis [32].

Some studies discussed the association between TGF-β1 polymorphisms and hepatocellular cancer risk. However, the results have been controversial [33].

So this study aimed to evaluate the role of gene expression of TGF- β1 in peripheral blood and its polymorphism 509C > T (rs1800469) in diagnosis of cirrhotic with HCC Egyptian patients chronically infected with HCV.

In this study, the mean age of patients with HCC was (62.0±8.7 years) (range 45-80 years) without significant difference among HCC and cirrhotic groups (P value = 0.13) **(Table 1)**. This result agreed with a previous study in 2017 [34] who reported that the age of the HCC patients (ranging from 24 to 83 years) with the mean age of (62.73±10.59 years). On the other hand, [35] reported that the age of HCC incidence was higher in Japan (70–79 years). This difference may be partially attributed to the difference in the risk factors distribution among Japanese patients with HCC, which was highly variable, depending on geographic region, race or ethnic group.

In the current study, HCC is presented more frequently in males than females with male to female ratio (1.33:1) **(Table 1)** with no significant difference between HCC and other groups**.** This male predominance came in agreement with a study in 2018 who reported that male/female ratio of HCC group was (1.3:1) without significant difference between other groups [36].

Several factors may explain male predominance in HCC as males are more likely to be infected with HCV and HBV, in addition to cigarettes smoking, and alcohol consumtion, testosterone rate has been shown to correlate with HCC indicating a probable role for the sex hormones in the development of HCC [37].

In the present work **(Table 2)**, there was statistically significant difference between HCC and cirrhotic groups regarding platelet count (P<0.001), this result was agreed with [5] who reported that there was significant difference between HCC and cirrhotic groups as regard platelet count (P<0.001).

In this study, there was statistically significant difference as regard serum creatinine level between HCC and control group and between HCC and cirrhotic group (P = 0.001, P = 0.024)

respectively **(Table 2)**, and this was agreed with a study in 2018 [38] who stated that there was significant difference between HCC group and healthy, chronic hepatitis C and LC as regard serum creatinine level. On the other hand [5] documented that, there was no statistically significant difference between HCC group and cirrhotic group as regards serum creatinine level, and this difference may be due to the difference in sample size as the previous study recruited larger number of patients (296 cases of HCC patients and 109 cases of cirrhotic without HCC patients) .

As regards AST level **(Table 2)**, there was statistically significant difference between HCC group and control group (P=0.027) and this result was agreed with previouse study in 2015 who stated that there was significant difference between HCC group and control group as regard AST level (P < 0.05) [11]**.**

Serum albumin level was statistically significantly lower in HCC group compared with the other groups (P value < 0.001) **(Table 2)** and this result came in agreement with [39] who reported significant lower level of serum albumin between HCC group and other study groups (P value <0.001).

In the current study, concerning INR level **(Table 2)**, there was statistically significant difference between HCC and cirrhotic groups (P=0.023), this result was agreed with the study in 2018 who reported that, there was significant difference between HCC and cirrhotic groups as regard INR level (P<0.001) [5] .

In the present study, there was statistically significant difference in AFP level between HCC group in comparison with control and cirrhotic groups (P value < 0.001) **(Table 2)**.

This finding agreed with [38, 40] who stated that, there was significant difference in AFP level between HCC group in comparison with cirrhotic, chronic hepatitis C and control groups (P value < 0.001) for all.

In the present study, most HCC patients were Child B (60%), followed by Child C (28.6%) then Child A (11.4%) with no statistically significant difference **(Figure 1).** Similar results were reported by the study in 2017 [34] who found that the majority of HCC patients were Child B (46.25%). On the other hand, [41] found that the majority of HCC patients were Child C.

In this study, regarding gene expression, there was a high statistical significant difference between control and cirrhotic with HCC groups (P<0.001) and between cirrhotic with HCC and cirrhotic without HCC groups (p<0.001)

(Table 3). This finding agreed with the study in 2008 who documented that, the value of TGF- β1 mRNA expression in patients with HCC was significantly higher compared to that in healthy volunteers (P < 0.000 1), and that the expression of TGF- β1 mRNA tended to be higher among patients with advancing histological aggressiveness. In general, the larger the tumor is, the higher the TGF- β1 mRNA level [42].

On the other hand, it was reported that TGF-β1 gene expression showed significant change among the HCC, LC, and control groups (p = 0.001). TGF-β1 gene expression in HCC patients was significantly lower than in LC patients (p = 0.042) and the control group (p = 0.001). In addition, TGF-β1 gene expression in LC patients was significantly lower than in the control group (p = 0.002). This difference in correlation may be due to the difference in the sample size which was smaller than our study (20 HCC, 20 LC and 20 healthy volunteers) [43]

In this study, there was no statistical significant difference between cirrhotic with HCC and cirrhotic without HCC groups and control group as regard TGF-β1 genotype and allele frequencies **(Table 4)**, and the most frequent genotype in cirrhotic with HCC group was CT (57.1%) followed by TT (22.9%) and CC (20%), and that the most frequent allele of HCC group was T allele (51.4%).

This finding was in agreement with [44] who stated that, there was no statistically significant difference between cirrhotic without HCC and HCC groups as regard TGF-β1 -509 genotypic frequency, with the most frequent genotype in HCC group was CT (23%) followed by TT (17%) then CC (14%). The most frequent allele in HCC group was T allele**.**

This also was coincided with a study in 2012 [45] who found that, there was no statistically significant difference between HCC and control groups as regard TGF-β1 -509 genotyping. The most frequent genotype in HCC group was CT (55.6%) followed by CC (33.3%) then TT (11.1%)**.**

Also, [46] reported that, the TGF-β1 -509 CC genotype was associated HCC in a Chinese population related to HBV infection. In another study in 2005 [47] reported that, the C allele of TGF-β1 -509, but not the T allele, might play an important role in the progression of liver cirrhosis.

On the other hand, a study in 2015 stated that TGF-β1 -509 gene polymorphism is associated with the risk of HCC in patients with chronic HCV infection in a Chinese Han population. The risk of

HCC was significantly higher among subjects with HCV infection carrying the TT genotype (P = 0.038) than patients carrying the CC genotype. The differences in these results may have been caused by different clinical characteristics as the previous study was conducted on 25.8% had portal vein thrombosis and 10.7% had lymph node metastasis, and those were excluded from our study. It is also could be due to ethnicity, designs and sample size, as it was relatively large (234 HCV without HCC + 159 HCV with HCC and 375 healthy volunteers) [11]**.**

Also, a previose study in 2016 [48] reported significant association of TGF-β1gene C-509T polymorphism with hepatocellular carcinoma.

Regarding Gene expression according to different frequent genotypes in HCC group. In this study, the highest gene expression was with TT genotype patients (P < 0.001) **(Table 5)**. This finding disagreed with [46] who stated that TGF-β1 Gene expression levels were significantly higher in CC patients than TT patients or patients carrying at least one T allele (P = 0.0002 or 0.006). This difference in results may be due to difference in ethnicity as the previous study was done on Chinese patients, large number of their sample size (575 patients and 299 healthy volunteers) and due to difference in etiology of HCC patients, as they included chronically infected with HBV not HCV .

In the present study, AFP sensitivity and specificity in the prediction of HCC were (82.9% and 84%) respectively **(Table 6)**. A previous study in 2014 [43] reported slightly lower sensitivity and higher specificity of AFP for discrimination between HCC and LC were (65% and 95%) respectively, this difference in sensitivity and specificity may be due to difference in the sample size which was smaller than our study (20 HCC, 20 LC and 20 healthy volunteers). On the other hand a study in 2017 reported slightly lower sensitivity and much lower specificity (72% and 43%) respectively, this difference in sensitivity and specificity may be due to difference in the selection of patients, as the previous study involved patients with vascular invasion and this was excluded from

our study and also may be due to different sample size, as he conducted his study on larger sample size (120 HCC, 30 LC and 30 healthy volunteers) [49].

In the current study, the sensitivity and specificity of gene expression in the prediction of HCC were (88.6% and 74%) respectively **(Table 6)**. A previous study in 2014 [43] showed the same

specificity (75%) but lower sensitivity (65%) for TGF-β1 gene expression to distinguish HCC and LC and they concluded that the combination of TGF-β1 gene expression and AFP level could be better.

In the current study, factors possibly associated with the development of HCC were assessed by univariable regression analysis compared with non HCC groups. These factors included age > 58 years, creatinine level > 1.3 (mg/dl), serum albumin level < 2.5 (g/dl), ESR > 80, AFP ≥ 41 (ng/ml) and level of gene expression ≥ 1.85 fold increase.

This was agreed with a study in 2018 who documented that, highest risk for development of HCC by binary logistic regression for prediction of HCC cases were age more than 58 years, hypoalbuminaemia and increase level of AFP [5]. A previous study in

2018 documented that age ≥ 50 years correlated with increasing risk of HCC development by univariate analysis of *TGF-β1* gene expression and HCC risk in Egyptian patients with chronic hepatitis C. Moreover, there was no significant association between *TGF-β1* potential risk factors of HCC in cirrhotic patients [50]. Also, it was stated that decrease albumin levels remained significantly correlated with HCC development by univariate analysis [51].

In the present work, multivariable binary logistic regression analysis for prediction of HCC revealed that only AFP ≥ 41 (ng/ml), level of gene expression ≥ 1.85 fold increase were significant independent predictors of HCV- related HCC **(Table 7).**

Up to our knowledge, no literature discussed these parameters as predictors for HCC but some studies reported that, AFP level was an independent risk factor associated with tumor differentiation, TNM stage, tumor size, and survival of patients with HCC [52] and that, the serum level of TGF-β1 was a significant independent prognostic factor of HCC [53].

Conclusion:

This study concluded that, there was significant association between *TGF-*

polymorphism −509C/T and HCC risk, with high frequency of CT genotyping in HCC group.

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