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TGF-1 mRNA, AFP-L3, and Annexin II in the Early and Late Detection of Hepatocellular Carcinoma: The Diagnostic Value

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Abstract

BACKGROUND: Alpha-fetoprotein (AFP) is the recommended screening biomarker for hepatocellular carcinoma (HCC), despite its drawbacks: AFP-negative HCC, poor specificity, and sensitivity. As a result, new HCC-sensitive and specific biomarkers are urgently needed.

AIM: This study aimed to determine the diagnostic value of transforming growth factor (TGF)-β1 mRNA and Annexin II in the early detection and follow-up of HCC.

PATIENT AND METHODS: This research involved 75 HCC patients (30 early and 45 late) and 75 liver cirrhosis (LC) patients (all patients have HCV), and 75 healthy individuals as controls. Reverse transcription polymerase chain reaction measured TGF-β1 mRNA. Enzyme-linked immunosorbent assay ELISA measured Annexin II, AFP-L3, and AFP.

RESULTS: Annexin II was a biomarker with a significant difference between the LC and early HCC groups. TGF-β1 mRNA showed a significant difference when the LC group was compared to the control group and the late HCC group.

CONCLUSION: Annexin II has better sensitivity and specificity for early HCC detection than AFP, and TGF-β1 mRNA can be used for the assessment of the degree of HCC, and TGF-1 signaling inhibitors may be a possible new treatment choice for HCC.

Introduction

According to the World Health Organization (WHO), hepatocellular carcinoma (HCC) is the fifth most prevalent form of liver cancer and ranks second in cancer-related deaths worldwide [1], [2]. However, according to Egypt's National Cancer Registry Scheme, HCC epitomizes significant public health, representing the first and second most prevalent cancers in men and women [1]. There is a dispute among the guidelines that have been found concerning alpha-fetoprotein (AFP) to complement abdominal ultrasonography (US) in detecting HCC. Since it is asymptomatic in the early stages and has limited therapeutic choices, HCC has a low survival rate [3]. The addition of AFP to US research significantly improves the sensitivity of HCC identification [4]. For tumors with a diameter less than 3 cm and tumors with a diameter greater than3 cm, the susceptibility to AFP varies from 25% to 50% [5]. False-positive AFP may be present in liver cirrhosis (LC) (11%), chronic hepatitis (15%), and other tumor forms (e.g., germ cell tumors), reducing the accuracy of AFP tests for HCC [6]. Due to the high prevalence of obesity (30%) and non-alcoholic

fatty liver disease among Egyptians and the anticipated decrease in HCV prevalence due to the Egyptian national campaign to eradicate HCV infection, it is expected that the HCC epidemiology in Egypt will change with respect to non-alcoholic steatohepatitis [7]. Consequently, the US's efficiency and sensitivity in these patients may be impaired, reducing HCC surveillance effectiveness [3]. On the other hand, this move would benefit AFP because false-positive cases would be diminished so that cutoff values would be more relevant [4]. The poor sensitivity and accuracy of AFP remain its key problems. New markers with higher sensitivity and specificity are needed to gain from curative treatment options for early HCC and improve clinical results [5], [8].

AFP-L3 is a fucosylated form of AFP with a high affinity for lectin (Lens culinaris agglutinin [LCA]) and is only formed by malignant liver cells. Compared to total AFP levels, it is more specific for detecting HCC. Two such glycoforms are AFP-L1 and AFP-L2. People with benign liver disorders such as cirrhosis or chronic hepatitis have non-LCA-binding AFP-L1, whereas yolk sac tumors have intermediate LCA-binding AFP-L2 [9].

Annexin II is a 36 kDa Ca^--dependent phospholipid-binding protein in nearly all eukaryotic cell membranes. Annexin II has a role in calcium-dependent exocytosis, immune responses, calcium delivery, and phospholipase A2 regulation [10]. The major biological processes that Annexin II proteins regulate are fibrinolysis, lipid messenger-mediated signaling, mitogenic-mediated signaling, and cell-cell adhesions. In terms of Annexin II, mRNA, HCC, and protein levels were more significant in HCC tissues. In contrast, cirrhotic liver hepatocytes displayed reduced expression relative to malignant hepatocytes [11]. Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine that regulates cell growth and differentiation in various organs and cells. It also plays an important role in extracellular matrix formation, angiogenesis, carcinogenesis, and immune suppression. TGF-β signaling plays a biphasic role in the development of HCC, where modifications to its signaling tend to be quite complicated. However, they significantly affect molecular pathogenesis [12]. This research aims to assess the diagnostic value of new markers such as Annexin II, AFP-L3, and TGF-1 Mrna's I for early HCC detection and associate their AFP levels, verified by various international guidelines in HCC surveillance. Also in this research, we aimed to classify the most vital biomarker that can substitute AFP.

Subjects and Methods

Subjects

This cross-sectional study was conducted on 225 participants drawn from the Internal and Tropical Medicine Department's outpatient and inpatient clinics at Al-Azhar University Hospitals, Cairo, Egypt. The study population was divided into three groups: The first group, the LC group, consists of 75 patients with HCV-induced LC. The diagnosis of LC was made after a thorough review of the patient's medical records and a thorough clinical and laboratory examination. Abdominal ultrasonography (US) was performed on all LC patients to ensure clear HCC, and one-third had a triphasic computed tomography (CT) for suspected US nodules. The second group, HCC patients, included 75 patients with LC and HCC who had a history of chronic HCV infection. Complex imaging patterns observed in a triphasic CT scan or dynamic enhanced magnetic resonance imaging (MRI) were used to diagnose HCC. In addition, HCC staging was performed on some instances using the Barcelona Clinic Liver Cancer staging [13], and cirrhosis was evaluated using the Child-Pugh classification. The third group, the control group, consisted of 75 people who seemed to be in good condition and with no signs or history of liver disease. Table 1 lists the study populations, clinical characteristics, and demographic details. Exclusion criteria: Patients undergoing hepatic focal lesion ablative therapy, immunosuppressive therapy, interferon therapy, or being treated for pre-existing or chronic HCC and patients with extrahepatic malignancies were excluded from the analysis.

Methods

The study proposal was accredited by the Research Ethics Committee of the Faculty of Medicine for Girls, Al-Azhar University Cairo, Egypt, with reference No RHDIRB202108948. In addition, all subjects' informed consent was obtained after explaining the study's purpose, procedures, and possible benefits. All research participants underwent a thorough clinical examination and laboratory examinations such as INR, CBC, and liver function tests (albumin, total protein, bilirubin [direct and total], ALP, GGT AST, and ALT). According to the manufacturer's guidance, ELISA kits were used to test for AFP-L3, AFP, and Annexin II in patient serum samples (AFP-L3: Human Diagnostics, Magdeburg, Germany, AFP: Monobind Inc., California, USA, and Annexin II: Life Science Inc., Wuhan, China).

Evaluation of TGF-1 Gene Expression: Reverse transcription (RT) followed by polymerase chain reaction (PCR) represents a powerful tool for detecting and quantifying mRNA. RT-PCR initially involves RNA extraction, then reverse RNA transcription into a complementary DNA copy (cDNA) by the enzyme reverse transcriptase, and finally amplifies the cDNA by PCR thermocycling. Real-time RT-PCR (OR kinetic RT-PCR) is widely and increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range. RNA extraction was done using QIAamp RNA Blood Mini Kit (for total RNA purification from the whole human blood catalog number 52304 [QIAGEN, Germany]) and RNase-Free DNase Set (catalog number 79254, QIAGEN, Germany).

Table 1: Demographic and biochemical profile of the studied patients

Parameters	Control group (n = 75)	Patients groups		p-value
		HCC (n = 75)	LC (n = 75)	
Age (M ± SD)	42 ± 12[a]	59 ± 11[a]	41 ± 11[a]	> 0.5 ns
Gender				
Male	32 (64%)	35 (70%)	45 (75%)	> 0.5 ns
Female	18 (36%)	15 (30%)	15 (25%)	
Total bilirubin (mg/dl)*	0.42 b (0.30–0.59)	1.75a (1.22–3.08)	1.40a (0.84–4.26)	< 0.001 HS
Direct bilirubin (mg/dl)*	0.08[b] (0.05–0.10)	0.70[a] (0.37–1.79)	0.81[a] (0.57–1.91)	< 0.001 HS
ALT (U/l)*	15.0[b] (10.5–23.0)	43.0[a] (32.0–51.0)	32.00[a] (23.0–52.5)	< 0.001 HS
AST (U/l)*	17.00[b] (14.5–21.0)	64.00[a] (44.5–112.0)	55.0[a] (43.0–65.50)	< 0.001 HS
ALT/AST**	0.85 ± 0.23[a]	0.50 ± 0.16[b]	0.58 ± 0.22[b]	< 0.001 HS
Total protein (gm/l)*	7.00[a] (6.75–7.45)	7.20[a] (6.10–7.80)	7.10[a] (6.20–7.70)	> 0.5 ns
Albumin (g/dl)*	4.00[a] (3.85–4.20)	2.60[b] (2.20–2.80)	2.60[b] (2.25–2.85)	< 0.001 HS
Hb (g/dl) **	12.91 ± 1.55[a]	10.99 ± 2.74[b]	10.24 ± 2.29[b]	< 0.001 HS
WBCs count (10 ≥ /l) **	6.67 ± 1.61[a]	4.66 ± 1.79[b]	8.04 ± 2.79[a]	< 0.001 HS
Platelets count (109/l) **	245.04 ± 48.9[a]	86.72 ± 37.85[cc]	141.72 ± 58.96[bb]	< 0.001 HS
INR *	1.02 b (1.00–1.07)	1.72 a (1.45–2.07)	1.50[a] (1.29–1.74)	< 0.001 HS

*Data existed as median and IQR interquartile range (25–75 percentiles). **The data are presented as mean ± SD, HS: Highly significant, ns: No significance. Groups bearing the same letters are not significantly different from each other at $p > 0.05$.

Statistical methods

IBM SPSS (SPSS Inc., Chicago, IL) advanced statistics version 28 was used to analyze the data. The Chi-square test was used to investigate the relationship between qualitative variables. The Kruskal–Wallis test has been used to evaluate three groups of non-normally distributed quantitative results [14], [15]. First, heat maps Spearmen ranked correlation test. Finally, the receiver operating characteristics (ROC) curve was used to test tumor markers' diagnostic significance in HCC diagnosis and, if appropriate, to assess the best cutoff values.

Results

Clinical characteristics of the studied groups

The descriptions and clinical characteristics of the patients included in this study are shown in Table 1. The majority of the patients were male (80%). The study included 110 patients, 50 of whom had HCC and were between the ages of 48 and 70, 60 patients with LC who were between the ages of 30 and 52, and 50 healthy controls between the ages of 40 and 52. According to the liver biochemical profiles, there is no substantial difference between the LC and HCC groups in bilirubin (total and direct), AST, ALT, and albumin. However, the differences are highly significant when comparing each group to the control group ($p < 0.001$). Furthermore, there were no significant differences in the hematological profile (Hb, WBC, platelets, and INR) between the LC and HCC groups, but significant differences when comparing each group to the control group ($p < 0.001$).

Descriptive statistical analysis

After statistical evaluation, there was a statistically significant difference in tumor markers (AFP, FP-L3, Annexin II, and TGF-β1 gene expression) between the two studied groups (LC group, early and late HCC group) and the control group Annexin II was the only biomarker showing a significant difference when comparing the LC and early HCC groups. Nonetheless, the four markers showed a significant difference when the LC group was compared to the late HCC group (Table 2).

Correlation analysis

The AFP and AFP-L3 had a clear, strong positive correlation that was highly significant, and the four markers tested had a non-significant correlation that alternated. Furthermore, the only two markers that displayed a significant intermediate positive correlation with tumor size were AFP and AFP-L3 (Figure 1).

ROC curve

As regards discrimination of the late HCC group from the LC group, Annexin II had the highest sensitivity (100.00%), specificity (96.24%), and area under the curve (AUC) (0.9524, $p < 0.0001$), followed by AFP-L3 with 79.38% sensitivity, 89.86% specificity, and 0.808 AUC. The third place comes AFP with 77.78% sensitivity, 81.82% specificity, and 0.798 AUC ($p < 0.001$). TGF-β1 gene expression showed 73.00% sensitivity, 81.00% specificity, and 0.778AUC (p −0.042) (Table 3 and Figure 2). Although Annexin II was the only marker that showed a significant difference between the LC and early HCC groups, calculating sensitivity, specificity, and AUC were done for all the markers as other studies showed such significance (Table 3 and Figure 2).

Table 3: Sensitivity, specificity, and AUC of the four markers in early and late HCC. AUC: Area under the curve, NPP: Negative predictive value, PPV: Positive predictive value, CI: Confidence interval

Tumor markers	Cutoff point	Sensitivity	Specificity	+PV	-PV	AUC	95% CI
AFP	Early > 20	68.00%	88.00%	85.0	73.3	0.741	0.56–0.87
	Late > 200	77.78%	81.82%	63.6	90.0	0.773	0.62–0.92
AFPL3	Early > 4.1	69.23%	86.36%	62.5	84.2	0.745	0.66–0.93
	Late > 4.5	79.38%	89.86%	70.0	90.5	0.808	0.59–0.91
Annexin II	Early > 21.2	88.89%	93.91%	72.7	95.0	0.891	0.72–0.97
	Late > 24.1	100.00%	96.24%	90.0	100.0	0.932	0.86–1.00
TGF–β	Early≤0.37	61.5%	86.4%	72.7	79.2	0.698	0.54–0.85
	Late≤0.46	73.00%	81.00%	70.0	90.5	0.753	0.66–0.94

The AUC for all four markers (AFP, AFP-L3, Annexin II, and TGF-β1) is greater in late HCC (below)

Table 2: Descriptive statistics of the four tumor markers in the studied groups

Tumor marker	Control (n = 75)	LC (n = 75)	Early HCC (n = 30)	Late HCC (n = 45)	p-value
AFP	1.20[c] (1.10–1.50)	34.00[a] (1.25–69.00)	16.53[ab] (1.90–836.0)	323.55[b] (36.22–7189.75)	< 0.001 HS
AFP-L3 (ng/ml)	0.05[c] (0.045–0.080)	0.50[a] (0.15–3.45)	1.20[ab] (0.15–63.10)	37.80[b] (3.87–81.64)	< 0.001 HS
Annexin II (ng/ml)	8.050[c] (6.60–11.45)	39.35[b] (31.80–45.83)	113.40[a] (91.3–133.5)	132.95[a] (107.3–148.8)	< 0.001 HS
TGF-β1 gene expression (N-fold change)	0.480[a] ((0.29–0.53)	0.670[b] (0.29–0.81)	0.77[bc] (0.47–0.96)	0.94[c] (0.66–1.37)	< 0.001 HS

*Data existed as median and IQR interquartile range (25–75 percentiles). HS: Highly significant. Groups bearing the same letters are not significantly different from each other at p > 0.05.

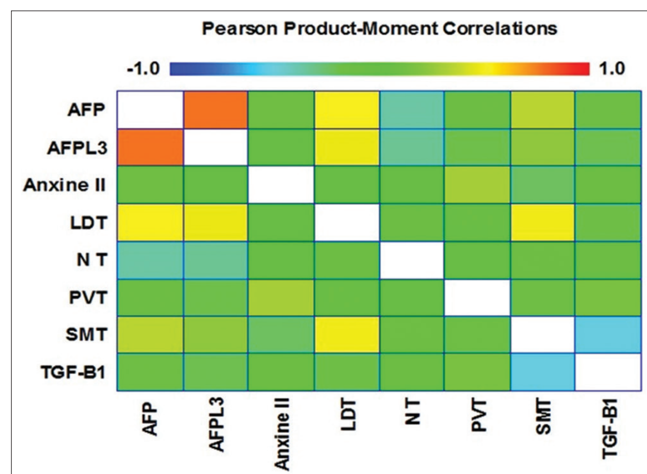

Figure 1: Heat map Pearson correlation shows the relationship between parameters. LDT: largest diameter of the main tumor (cm), NT: no of tumors, PVT: Pulmonary vein thrombosis SMT: Side of the main tumor

as compared to early HCC (above), with Annexin II showing the greatest AUC in both ROC curves.

Discussion

HCC is the most deleterious complication of LC [16]. Although HCC is often detected in the context of cirrhosis, cirrhosis is not a premalignant lesion in and of itself. Instead, the transition between cirrhosis and hepatocarcinogenesis develops simultaneously over the years to decades [16]. When comparing the HCC (early and late) and LC groups to the control group, the four markers used in this analysis indicated a significant difference (p < 0.001). The only marker that demonstrated a significant difference between the LC and the two HCC groups (early and late HCC) (p < 0.001) was Annexin II. On the contrary, the other three markers (AFP, AFP-L3, and TGF-1 mRNA) presented only a significant difference with the late HCC group (p < 0.001). This may imply that Annexin II is a promising tumor marker that can detect early HCC in patients with LC and HCV infection.

The studies of Zhang *et al*. [17] found that HCC tissues had higher expression of Annexin II mRNA and Annexin II protein levels and more elevated serum Annexin II levels than LC cases. Furthermore, higher Annexin II expression in HCC adjacent normal tissue was due to the neoplastic transformation phase and genetic alterations from hepatitis virus infection-induced long-term inflammation [17]. Annexin II and AFP had cutoff values of 18 ng/L and 50 ng/mL, respectively, with a sensitivity and specificity of 86.96%, 66.67%, 70.43%, and 73.08%, respectively. According to Shaker *et al.* [18], Annexin II had a sensitivity of 74% and a specificity of 88% at a cutoff value of 18 ng/mL. In comparison, at a cutoff value of 200 ng/dL, AFP included a sensitivity of 20% and specificity of 100%. El-Abd *et al.* [19] observed that at a cutoff value of 29.3 ng/ml, Annexin II's AUC was 0.910 (95% CI 0.84–0.97).

Variations in performance characteristics between this research and the other studies may be due to differences in pathological histories and HCC grades. In chronic HCV patients, AFP elevations may be due to both hepatocarcinogenesis and the inflammatory processes that occur concurrently over time. Zhang *et al.* [20], a recent Chinese meta-analysis report that examined 59 studies from various countries (including five Egyptian studies), addressed the debate about an acceptable AFP threshold for the diagnosis of HCC. In terms of sensitivity, accuracy, and AUC of summary ROC (SROC), the 400 ng/mL cutoff for AFP outperformed the 200 ng/mL cutoff. Sensitivity, accuracy, and the AUC of summary ROC were 32%, 99%, and 0.937, respectively, at a 400 ng/mL threshold (four studies), 49%, 98%, and 0.931, respectively, at a 200 ng/mL threshold (four studies), and 61%, 86%, and 0.833, respectively, at a 20–100 ng/mL threshold (46 studies). Lower sensitivities and higher specificities were obtained by increasing the AFP threshold and, considering their findings [20], proposed using the 20 ng/mL threshold in HCC surveillance due to increased sensitivity.

Many cancer development pathways, including epithelial-mesenchymal transition, migration, cancer growth, invasion, and susceptibility to radiotherapy, chemotherapy, and immunotherapy, are strengthened by annexin II [21]. In addition, Annexin II silencing has inhibited hepatoma cell tumorigenic capacities. Higher levels of Annexin II were shown to be associated with advanced clinicopathological characteristics and a lower overall 5-year survival rate in HCC patients, making it a possible prognostic biomarker and a therapeutic goal [22].

In the present study, early HCC patients had higher AFP-L3 values (median 1.2, IQR 0.15–63.10) than cirrhotic patients (median 0.50, IQR 0.15–3.45), but the difference was not statistically significant. Compared to cirrhotic patients, late HCC patients had highly significant AFP-L3 values (median 37.80, IQR 3.87–81.64). Furthermore, AFP-L3 levels were

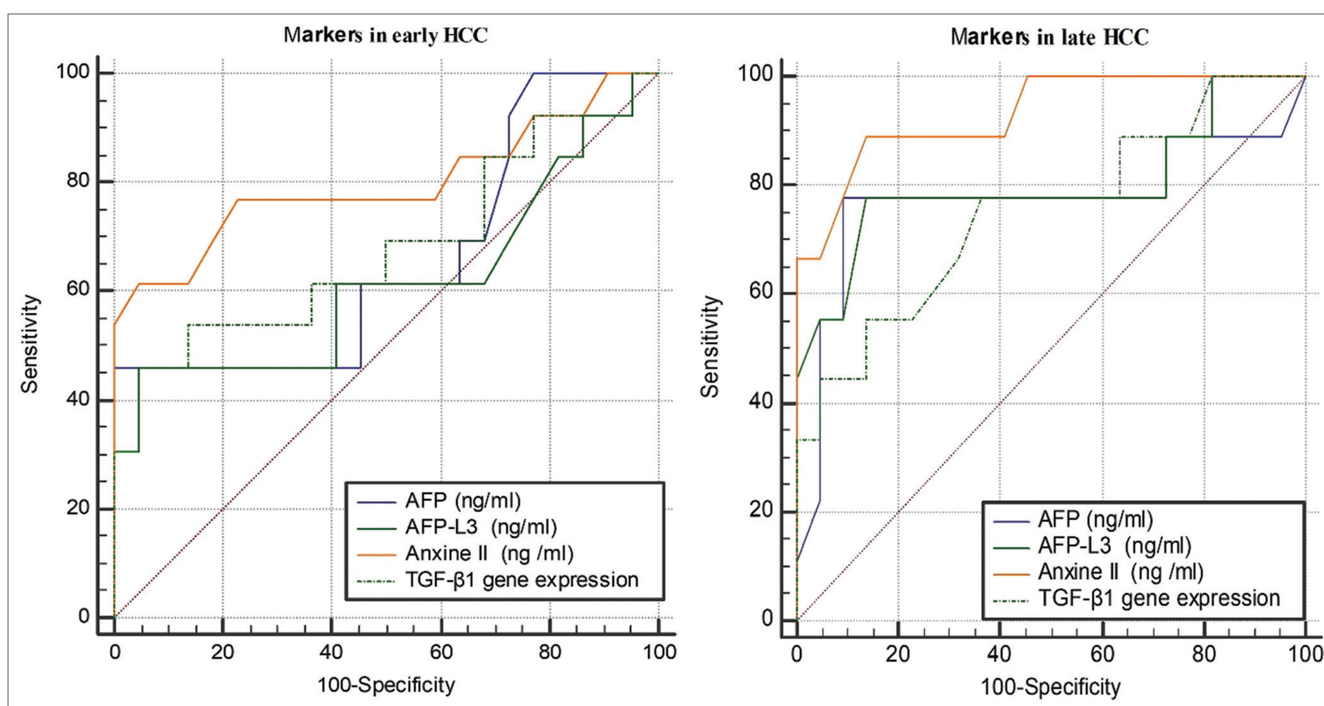

Figure 2: ROC curves of the four markers studied in early HCC (Right) and late HCC (left).

markedly higher in the LC group compared to the control group. The slight increase in AFP-L3 in the LC population, while remaining below the cutoff value for HCC diagnosis, may be clarified by the premalignant state of LC patients. Elevated serum AFP-L3 is more specific for HCC than overall AFP and has been observed in 35% of patients with small HCC of <2 cm [9]. MRI observed significant AFP-L3 elevation in several patients before the diagnosis of HCC. Chen *et al.* [23] conducted a new meta-analysis review and found that AFP-L3 performed better than AFP in the diagnosis of HCC, yielding results similar to the current study. These contradictory findings could be attributed to the use of different assay methods and to the heterogeneity of HCC in terms of etiology and pathological type.

In the current research, AFP-L3 was shown to have a strong positive correlation with tumor size. However, it demonstrated non-significant correlations between portal vein thrombosis and tumor number. The authors of Durazo *et al*. [24] found no correlation between AFP-L3 and vascular invasion, histological grade, or tumor size. AP-L3, on the other hand, was correlated with larger tumor size, metastasis, portal vein invasion, and faster growth. Furthermore, AFP-L3 may be able to predict the recurrence of HCC [22]. Compared to normal liver tissues, there is a significant difference in the expression of the TGF-1 gene in HCC. This may be explained by TGF-1's dual function in hepatocarcinogenesis (late tumor promoter and early suppressive) as well as the heterogeneity of HCC grades observed in various studies [25], [26]. Due to its early cytostatic and apoptotic abilities in tumor cells, the TGF gene can protect against tumor progression. Cellular resistance and changes in the tumor microenvironment, on the other hand, cause it to promote tumor growth [27]. In this study, TGF-1 mRNA levels were significantly higher in the late HCC group than in the LC and control groups. Furthermore, TGF-1 mRNA levels were higher in the early HCC group relative to the LC group, but the disparity was not statistically significant (Table 2). This was in accordance with Teama *et al.* [28] who found elevated TGF-1 mRNA levels in patients with LC and Peng *et al.* [29] who found elevated TGF-1 mRNA levels in patients with HCC patients compared to normal controls, with higher values associated with advanced histological aggressiveness.

This result was related by the authors of Dong *et al*. [30] to differences in cellular sensitivity to TGF-1 growth inhibition, beginning with early higher cellular sensitivity to low TGF-1 mRNA values and ending with late lower cellular sensitivity to advanced tumor stages.

This finding is contrary to Farid *et al.* [31] who found that TGF-1 mRNA was slightly lower in the HCC group versus in the LC and control groups. TGF-1 mRNA was found in HCC (advanced and early), cirrhotic, dysplastic, and mild liver [32]. TGF-1 mRNA levels were shown to be downregulated from the cirrhotic to the dysplastic phase, supporting the hypothesis that TGF acts as a tumor suppressant in normal epithelial cells and that TGF signal deficiency in the early stages contributes to tumor outgrowth and development. Similarly, Deng *et al.* [33] discovered a substantial decrease in TGF-1 mRNA expression in malignant liver tissue instead of neighboring normal liver tissue ($p = 0.033$). Lin *et al.* [32] used immunohistochemistry to prove that the TGF-1 protein expression levels of

HCC tissue were lower than that of neighboring normal liver tissue ($p < 0.001$).

TGF-1 mRNA expression levels did not correlate with specific markers in the present study, such as tumor size or the presence of multiple tumors. Findings are supported in [28], where non-significant associations were observed between AFP levels and tumor size, grade, and TGF-1 mRNA. Many potential TGF-1 inhibitors are currently being evaluated in phase I/II clinical trials [32], [34] because of high TGF-1 mRNA expression levels in patients with progressive histological aggressiveness and high AFP levels [27].

In HCC therapy, the combination of TGF-1 receptor kinase inhibitors and the anti-PD-1 or sorafenib immune checkpoint inhibitor anti-PD-1 or sorafenib has been studied, with a longer average survival in responders and a more significant survival advantage for patients with elevated AFP levels [35].

Conclusions

The current findings indicate that Annexin II has better sensitivity and specificity for early detection of HCC than AFP. Therefore, TGF-1 signaling inhibitors may be a possible new treatment choice for HCC.

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Authors' Contributions

Conceptualization, E.M.I.Y, N.E-L, R.M.E.T, A.A.E, and M.A.K, data collection, E.M.I.Y, A.A.E E.S.M.B, Y.A.A, A.E.E, F.S.M, and S.M.F, methodology, R.M.E.T, E.M.I.Y, E.S.M.B, M.A.K, A.A.E, G.F.E, A.E.A, N.S, and S.M.H.M, writing, E.M.I.Y, N.E-L, A.A.E, and M.A.K, writing – original draft preparation, E.M.I.Y, R.M.E.T, N.E-L, A.A.E, M.A.K, S.M.F, and S.M.H.M, writing – review and editing, E.M.I.Y, E.A.K, A.A.E, E.S.M.B, Y.A.A, A.E.E, F.S.M, N.S, G.F.E, M.R.S, S.M.F, and S.M.H.M, and project administration, E.M.I.Y, E.A.K, and Y.A.A.

All authors have read and agreed to the published version of the manuscript.

Declarations

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Ethics approval

The study protocol was approved by the Ethics Committee of the Faculty of Medicine for Girls, Al-Azhar University Cairo, Egypt, with reference No RHDIRB202108948.

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