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Interaction of peripheral $CD4^+CD25^+CD127^-$ Tregs with prolactin in HCV hepatocellular carcinoma: oncogenic or immunogenic mechanisms

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Abstract

Background and objective There is little and conflicting data about the peripheral $CD4^+CD25^+CD127^-$ Tregs in patients with hepatocellular carcinoma (HCC) of various etiologies. The expressed membrane-bound transforming growth factor (mTGF-β1) on these Tregs is a marker of their suppressive function. Prolactin suppresses Tregs function in healthy subjects but enhances local Tregs in breast cancer. Our study is the first to assess the frequency and function of $CD4^+CD25^+CD127^-$Tregs and their association with clinicopathological features and staging in HCV-related HCC and to determine whether prolactin acts as an oncogenic growth factor or participates in the regulation of the immune response mediated by peripheral Tregs. In patients with HCV- elated HCC, HCV-cirrhotic patients, and healthy subjects, we measured the frequency of peripheral traditional $CD4^+$ $CD25^+$ Tregs and well-characterized $CD4^+CD25^+CD127^-$Tregs and their mTGF-β1 using flow cytometric analysis and measured serum prolactin level.

Results The frequency of $CD4^+$ $CD25^+$ and $CD4^+CD25^+CD127^-$ Tregs was comparable between HCC and cirrhotic patients and healthy subjects. Serum prolactin and mTGF-β1 on traditional and $CD4^+CD25^+CD127^-$ Tregs were significantly higher in HCC and cirrhotic patients than healthy subjects with an insignificant difference between HCC and cirrhotic patients. Roc curve analysis revealed that cutoff value for mTGF-β1 on Tregs ≥ 13.5% is a good specific (87%) but low sensitive (54%) test in discriminating HCC patients from healthy subjects. The frequency of Tregs and mTGF-β1 were not correlated to clinicopathological characteristics or staging of HCC. Prolactin was higher in the multifocal lesions and negatively correlated to expressed mTGFβ1. The expressed mTGF-β1 was positively correlated with hemoglobin and alanine transaminase. The traditional Tregs was positively correlated with hemoglobin and albumin.

Conclusion mTGFβ1, as a marker for suppressive function of peripheral CD4 + CD25 + CD127-Tregs, has a diagnostic role in discriminating HCV-related HCC patient from healthy subjects, unfortunately not from HCV-related cirrhotic patients. Serum prolactin has an oncogenic role as it is correlated to multiple focal lesions. It also impedes the suppressive function of peripheral Tregs as an immunogenic role. mTGF-β1 is related to hemoglobin and hepatic inflammation.

Keywords HCV-related hepatocellular carcinoma, T-regulatory cells, Prolactin, Peripheral CD4 + CD25 + CD127- Tregs, Membranous TGF-β1

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Background

Globally, hepatocellular carcinoma (HCC) is one of the most prevalent cancers and is the second-leading reason for cancer mortality [1]. In Egypt, hepatitis C virus (HCV) is the most common cause for HCC development on top of liver cirrhosis [2]. The development of HCC is a complex multistep issue [3]. One of the mechanisms underlying hepatocarcinogenesis is an immunological escape. Stromal cells including immune cells, extracellular matrix, cytokines, and chemokines form the extra-parenchymal tumor microenvironment in HCC. Immune cells, including antigen-presenting cells and effector lymphocytes, primarily intensify a replay against transformed cells, but with sustained inflammation, there is the recruitment and accumulation of T regulatory cells (Tregs) in the tumor tissues [4]. Tregs are a subpopulation of T cells with immune suppressive regulatory function which can impede this replay via suppression anti-tumor effector T-cell activation and proliferation, allowing tumor cells to proliferate, and facilitating tumor immune escape [5]. Tregs were originally referred to as CD4 + CD25 + Tregs because of their constitutive expression of the surface CD4 and CD25 antigens. Traditional CD4 + CD25 + Tregs, a subset of T lymphocytes that naturally exist in the immune system and make up 5–10% of CD4 + T cells, suppress both CD4 + and CD8 + T cell activation and proliferation in both vivo and vitro through a variety of mechanisms, including cell-to-cell contact and immune-suppressive cytokines like TGF-ß1 [6] The CD4 + CD25 + phenotype is insufficient to define Tregs because CD25 is not restricted to Tregs and is expressed by a much more diverse T cell population, such as activated CD4 T cells [7]. Furthermore, the use of CD4 + CD25 high as a marker of Tregs is controversial for a variety of technical reasons [8]. However, CD4 + CD25 + Tregs are still widely used in human studies. The search for a unique marker that accurately and specifically identifies Tregs has been ongoing. Foxp3, a nuclear transcription factor, is a more specific marker for Tregs. Unfortunately, it cannot be used to separate human Tregs for functional studies because it is a nuclear marker [9, 10]. Many researchers now accept the CD4 + CD25 + CD127- Tregs phenotype as a surrogate marker for Tregs for a variety of reasons, including (a) the absence of CD127 as a surface marker distinguishes CD4 + CD25 + Tregs from activated CD4 T cells, (b) it can distinguish Tregs with either high or low CD25 expression, and (c) it can distinguish Tregs with potent suppressive function more efficiently than CD4 + CD25 + Tregs [10, 11]. Furthermore, CD4 + CD25 + CD127- Tregs express Foxp3 and represent a phenotypically pure population. CD127 expression is inversely related to Foxp3 expression and is helpful in isolating these Tregs of interest because, unlike Foxp3, CD127 is surface expressed and can be sorted without interrupting the integrity of the plasma membrane, enabling further experimentations [12]. Finally, in clinical practice, CD4 + CD25 + CD127 low/- Tregs are presumed surrogate markers for recognizing Tregs as they are more specific and precise than the combined markers CD39, CD73, and CD25 + [13]. The suppressive function of Tregs is mediated by cell–cell contact or the expression of membranous transforming growth factor-1 (mTGF-ß1) [14]. Tregs and their associated factors such as metabolites and secreting cytokines mediate the immune tolerance of the tumor microenvironment in HCC. Therefore, targeting Tregs and blocking their mediated factors may prevent HCC progression [15]. TGF-ß1 aids in the formation of a favorable microenvironment for tumor development and progression via many signaling pathways involving Tregs, cancer-associated fibroblasts, and inflammatory mediators [16]. While the local recruitment of Tregs in HCC tissue is well known [17, 18], there is little and conflicting data on the peripheral expression of CD4 + CD25 + CD127-Tregs in HCC patients. Both upregulation and downregulation of these Tregs were observed in hepatitis B virus (HBV)–HCC patients compared to healthy subjects. Furthermore, the high frequency of these Tregs was linked to HBV infection. Circulating Tregs were not only more frequent in HBV-HCC patients, but they were also more suppressive and released greater TGF-1 than non-HBV HCC patients (alcoholic as well as non-alcoholic steatohepatitis) [19, 20]. So, the aetiology of HCC may alter Tregs expression and suppressive function. To the best of our knowledge, no study has looked at peripheral CD4 + CD25 + CD127-Tregs in HCV-related HCC.

Prolactin (PRL) is a protein hormone as well as a pleiotropic cytokine. Aside from its classic endocrine functions, it participates in the organization of the innate and adaptive immune responses and acts as an autocrine and/or paracrine growth factor in different tissues and malignancies [21, 22]. Prolactin can be produced extra-pituitarily by immune cells such as B lymphocytes and T lymphocytes, in addition to the lactotroph cells of the anterior pituitary gland, which are the main synthesized cells. PRL mainly prevents the negative selection of auto-reactive B lymphocytes. It also enhances the activation, differentiation, and proliferation of T cells [21]. PRL receptor expression is constitutive in Tregs but not in T-effector cells, while the expression of PRL is constitutive in both populations. PRL inhibits the suppressive function of Tregs in healthy individuals as it downregulates circulating CD4 + CD25 + CD127-Tregs' function [23]. Also, PRL

has an oncogenic effect on cancers of various tissues in an autocrine or paracrine manner [22]; PRL receptors were first identified in the liver as the main target organ and then were identified in HCC tissue [24, 25]. In animal studies, prolactin may have oncogenic and protective roles in HCC development [26, 27]. It may mediate its oncogenic role via the JAK2-STAT3 pathway [28]. The serum PRL level was found to be significantly higher in HCC patients with HBV aetiology [29], but results in HCV-HCC patients were unmatched [30–32].

This is the first study to look at the frequency of peripheral blood CD4 + CD25 + CD127-Tregs and identify the expression of mTGF-ß1 on these Tregs as a marker for their immunosuppressive function and possible effects on HCC susceptibility and clinicopathological features and to look at serum PRL levels and see if PRL plays a role in the regulation of the immune response mediated by peripheral Tregs in patients with HCV-related HCC.

Subjects and methods

Study population

The current cross-sectional case–control study enrolled 48 HCV-cirrhotic patients with HCC (males/female [m/f]: 36/12) who constructed the HCC group and 48 HCV-cirrhotic patients (m/f: 32/16) who represented the cirrhotic group. They have recruited from inpatient and outpatient clinics, the internal medicine department, the faculty of medicine, and the Minia University Hospital. The study was conducted in collaboration with the flow cytometric unit at the South Egypt Cancer Institute and Assuit University from June 2015 to June 2016. Forty-eight healthy age and sex-matched subjects (m/f: 30/18) served as the control group. The study protocol was approved by the local ethical scientific committee at Minia University. Informed medical consent was obtained from all participants before the study.

Study design

All participants were subjected to a thorough history and clinical examination. The diagnosis of chronic HCV infection was based on the detection of anti-HCV antibodies for ≥ 6 months and the presence of HCV RNA using PCR. Liver cirrhosis was diagnosed according to abdominal ultrasonography and basic laboratory investigations. The severity of liver dysfunction was graded using the Child–Pugh classification [33]. HCC was diagnosed using dynamic imaging CT or MRI in accordance with the European Association for the Study of the Liver (EASL) guideline 2012 [34]. The staging of HCC was based on tumor node metastasis (TNM) system, according to the American Joint Committee on Cancer (AJCC) [35]. Tumor size, number of focal lesions, and portal vein thrombosis were evaluated as tumor characteristics. Exclusion criteria were the presence of malignancy elsewhere, diabetes mellitus, endocrine disorders, pregnancy or lactation, any organ dysfunction, recent infection, alcohol intake, or any local or systemic treatment for HCC, other causes of cirrhosis, or co-infection with HBV and/or HIV.

Biochemical assays

Fasting venous blood samples (7 ml) were collected. The prepared samples were used for the measurement of routine laboratory investigations. Viral markers for chronic viral hepatitis and HIV were determined. Hepatitis C virus antibodies and HIV virus antibodies were qualitatively detected using third-generation and fourth-generation enzyme-linked immunosorbent assays (ELISA), respectively (BIO ELISA HCV kit and BIO ELISA HIV kit; BIOkit, a Werfen company, Barcelona, Spain). Hepatitis B surface antigen was detected by qualitative ELISA kits (Sanofi Diagnostic Pasteur, Marne-la Coquettee, France). Positive cases for HCV antibodies were confirmed using real-time PCR using the ABI 7300 prism® (Applied Biosystem, Foster City, USA). Stored serum samples at 20 °C were used for assaying serum PRL using the quantitative sandwich enzyme immunoassay technique (Biocompare, South San Francisco, CA, USA). All assays were performed according to the manufacturer's instructions.

Flow cytometric analysis for peripheral blood CD4, CD25, CD127 Tregs, and TGF-β expression

A sterile tube containing an anticoagulant, ethylene diamine tetraacetic (EDTA) salt (Becton Dickinson ®, UK), was used to draw 3 mL of the venous blood. The samples were kept at room temperature (18–25 C), and flow cytometry was used to detect CD4, CD25, CD127, and TGF-expression within 24 h of venipuncture. Phenotypic analysis of peripheral blood mononuclear cells (PBMCs) in the whole blood samples was performed on a fluorescence-activated cell sorter (FACS Calibur™ flowcytometer, 2 lasers, 4 colors, Becton Dickinson Biosciences, San Jose, California, USA) using a set of fluorochrome-labeled monoclonal antibodies against surface CD4, CD25, CD127, and membrane bound TGF-β [36]. The used monoclonal antibodies are –APC-conjugated -CD4, FITC-conjugated anti-CD25 (BECKMAN COULTER, USA, clone 13B8.2 & clone B1.49.9, respectively), PE-conjugated anti-TGF-β1, and PerCP-conjugated anti-CD127 (BD-Biosciences, USA, cloneTW4-9E7 & clone H1N7RN, respectively).

Statistical analysis

The collected data were tabulated and analyzed using SPSS version 17 software. Categorical data were presented as numbers and percentages, whereas quantitative data were expressed as the median and 25–75% interquartile range. A comparison of continuous data between more than two groups was made using the Kruskal–Wallis test followed by the Mann–Whitney test. The *c*2-test and Fisher's exact test were applied for comparison between categorical data. The correlations between different parameters (non-parametric) were assessed using the Spearman test. The diagnostic utility of mTGF-ß1 on CD4 + CD25 + CD127-Tregs in HCC patients versus healthy subjects was assessed using a receiver operating characteristic (ROC) curve analysis. The area under the ROC curve (AUC) and the optimum cutoff value for specificity and sensitivity were calculated using the Youden index (J) test. $P < 0.05$ was considered significant.

Results

Clinical and laboratory characteristics of the studied groups

The detailed laboratory characteristics and the comparisons between the HCC, cirrhotic, and healthy subject groups are shown in Table 1. The HCC group was matched to the cirrhotic group with regard to the hematological data and most studied hepatic parameters and the severity of liver disease (Child class). However, the cirrhotic group had significantly higher international normalized ratio and lower serum albumin. The HCC and cirrhotic groups had significantly higher PRL levels than healthy subjects with insignificant differences between the HCC and cirrhotic patients ($p < 0.001$, $p = 0.002$, respectively).

Flow cytometric characteristics of studied groups

In the HCC, cirrhotic, and healthy groups, the frequency of traditional Tregs (CD4 + CD25 +) and a newly identified subset of Tregs, CD4 + CD25 + CD127- Tregs, was matched. Furthermore, the HCC and cirrhotic groups had significantly higher mTGF-beta frequencies on both CD4 + CD25 + Tregs and CD4 + CD25 + CD127- Tregs than healthy subjects (p 0.001 for all), as well as an insignificant difference in cytometric data between the two diseased groups (Table 1). Gating of peripheral blood monocular cells, identification of CD4 + CD25 + Trgs, and expression of mTGF beta on CD4 + CD25 + CD127-Tregs using flow cytometry are shown in Fig. 1 (A, B, and C, respectively).

Diagnostic performances of mTGF-ß1on CD4 + CD25 + CD127- Tregs

ROC curve analysis showed that the frequency of mTGF-ß1 on CD4 + CD25 + CD127-Tregs is a

Table 1 Demographic, laboratory, and flow cytometric data of the studied groups

Variables	HCC group ($n = 48$)	Cirrhotic group ($n = 48$)	Healthy group ($n = 48$)	*P* value
Age (years)	54 (51.5–59.2)	55 (50–58)	55 (49–59)	0.86
Male gender (%)	36 (75%)	32 (66.7%)	30 (62.5%)	0.88
Hemoglobin% (gm/dl)	10 (9.1–11.3) ***	10.3 (8.9–10.9) ***	14.5 (12.7–15.2)	**< 0.001**
White blood cells count (× 103	6.8 (5.2–9.2)	6.5 (4.1–8.4)	6.6 (5.6–8.2)	0.28
Platelet count (× 103)	107 (93–147) ***	103 (55–137) ***	258 (205–305)	**< 0.001**
Alanine aminotransferase (U/l)	47 (25–62)	32 (25–51) **	21 (14.5–33)	**< 0.001**
Aspartate aminotransferase(U/l)	67 (37–89)	61 (37–77) ***	23 (17–34)	**< 0.001**
Albumin (gm/dl)	2.7 (2.3–2.9) ‡***	2.4 (2.1–2.8) ***	4.2 (3.8–4.5)	**< 0.001**
Bilirubin (mg/dl)	1.9 (1.1–3.8) **	2.5 (1.4–4.8) ***	0.70 (0.40–0.90)	**< 0.001**
International normalized ratio	1.6 (1.3–1.8) ‡‡ ***	1.8 (1.5–2.3) ***	1 (1–1.1)	**< 0.001**
Creatinine (mg/dl)	0.97 (0.7–1.3)	0.95 (0.8–1.2) *	0.90 (0.8–1.05)	0.83
Child class A/B/C(n)	6/18/24	2/16/30	–––	
Serum Prolactin (ng/ml)	20 (12–50) ***	25 (12–45)**	10 (5.1–30.9)	**< 0.001**
Frequency of CD4 + CD25 + Tregs (%)	4.5 (1.2–9.4)	4.91 (2.1–13.5)	6.8 (1.6–8.4)	0.47
Frequency of D4 + CD25 + CD127-Tregs (%)	64.9 (34.6–80.7)	67.6 (43.3–81.3)	71.4 (57.8–84.4)	0.29
Frequency of TGFβ1/CD4 + CD25 + Tregs (%)	28.3 (15.2–54.4) ***	25 (9.6–57.7) ***	10 (5.1–30.9)	**< 0.001**
Frequency of mTGFβ1/CD4 + CD25 + CD127- Tregs (%)	15.5 (6–30.9) ***	20 (8.9–35.4)***	4.9 (1.9–12.2)	**< 0.001**

HCC hepatocellular carcinoma, *CD* cluster differentiation, *mTGFβ*$_1$ membrane bound transforming growth factor β$_1$, *Tregs* T regulatory cells. Gender and child class are expressed as number (percentage) and compared by chi-square test. Quantitative data are expressed as median and interquartile (25–75%) and compared using the Kruskal–Wallis test between the three groups followed by the Mann–Whitney test between each two groups. $P < 0.05$ is considered to be statistically significant. Significant difference when HCC patients compared to cirrhotic patients $p < 0.05 = ‡$, $p < 0.01 = ‡‡$. Significant difference when either HCC or cirrhotic groups compared to healthy subjects $p < 0.05 = *$, $p < 0.01 = **$, and $p < 0.001 = ***$

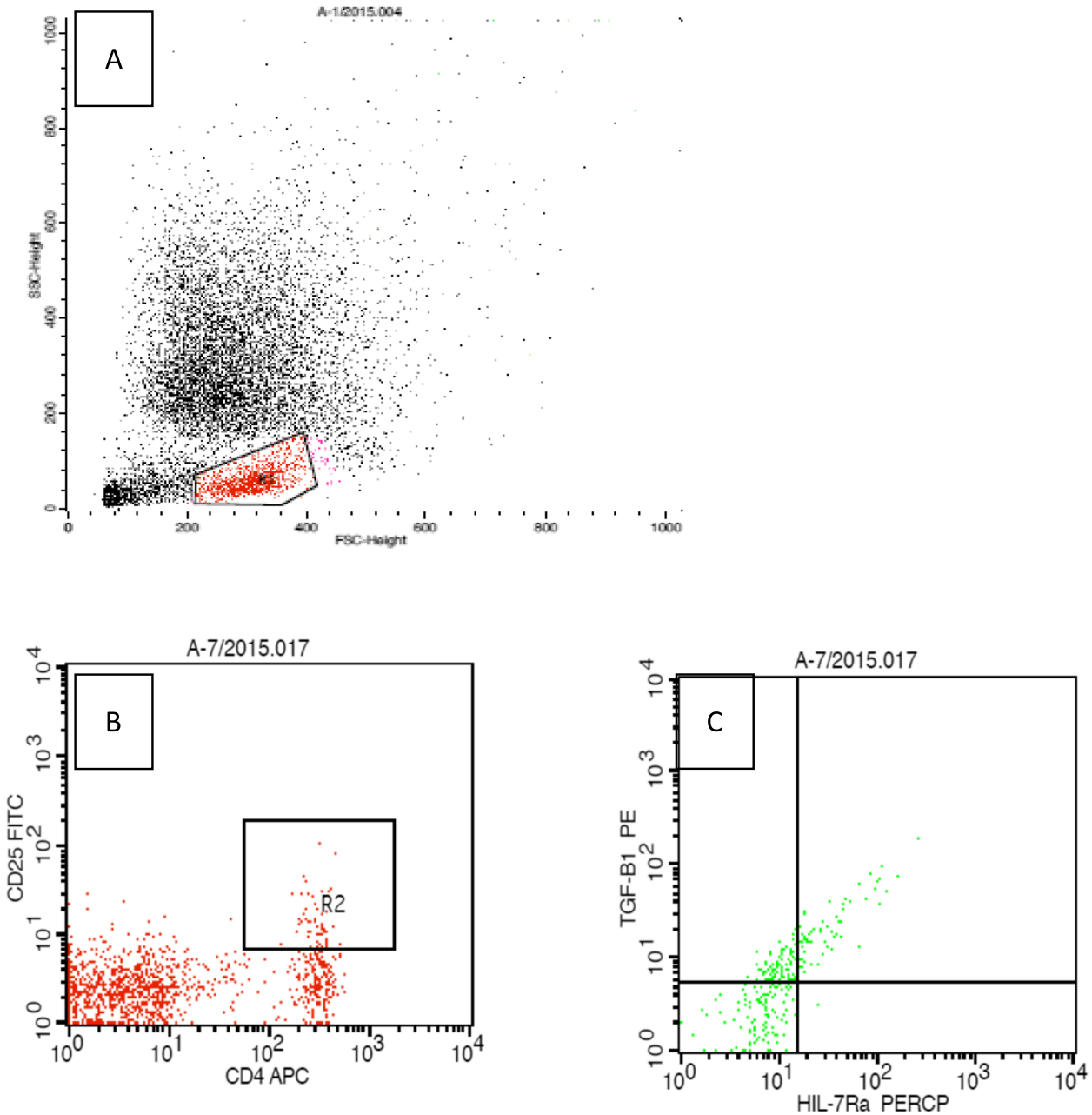

Fig. 1 **A** Forward (FSC) and side (SSC) scatter histogram was used to define lymphocyte population in peripheral blood mononuclear cell. **B** The CD4 + CD25 + cells (R2) was assessed within the lymphocyte population (R1) by their co expression of CD4 + and CD25 + on the cells. **C** The expression of mTGF-ß1 was assessed on Tregs CD4 + CD25 + CD127-. mTGF-ß1 on CD4 + CD25 + CD127- Tregs in upper right quadrant and mTGF-ß1 on CD4 + CD25 + Tregs in upper left quadrant

useful specific biomarker to separate HCC patients from healthy subjects with an AUC of 0.71 (95% confidence interval (CI) 0.60 to 0.81, p 0.001) (Fig. 2). At a cutoff value of ≥ 13.5% for the frequency of mTGF-ß1 on CD4 + CD25 + CD127-Tregs, the optimal specificity and sensitivity were 87% and 54%, respectively.

Morphological features and staging of HCC and association with circulating Tregs and serum prolactin

We found tumors ≥ 5 cm in diameter in 62.5% (30/48), multiple tumor lesions in 56.25% (27/48), and portal vein thrombosis (PVT) in 54.2% (26/48) of HCC patients. There was no evidence of a lymph node or distant metastasis. The

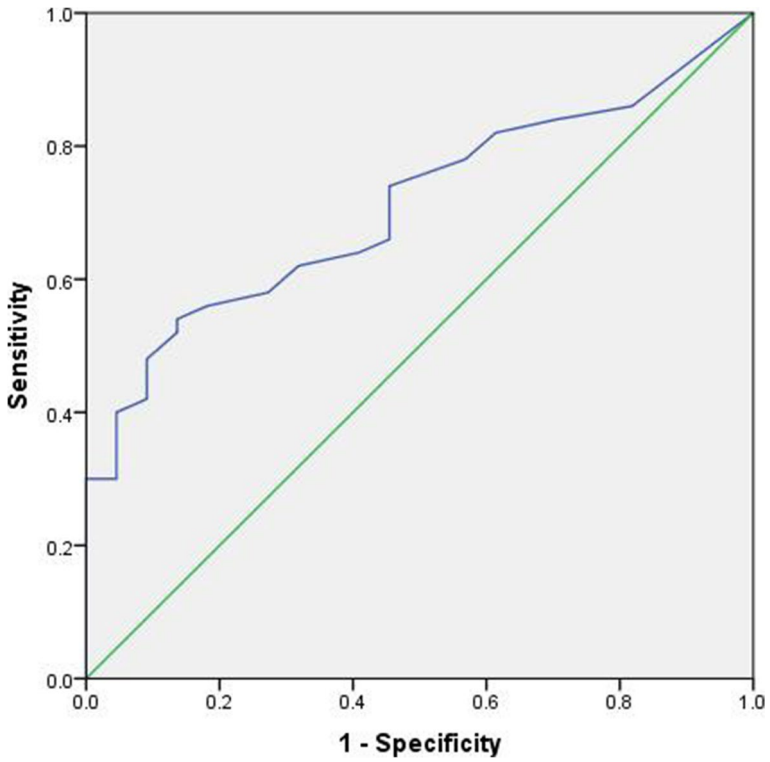

Fig. 2 The ROC curve analysis for the diagnostic values of mTGF-ß1on CD4 + CD25 + CD127- Tregs in discriminating HCC patients from healthy subjects. ROC curve identified an optimal cutoff value of ≥ 13.5 for mTGF-ß1on CD4 + CD25 + CD127- Tregs to discriminate HCC patients from healthy subjects. The area under curve (AUC) is 0.71 Sensitivity and specificity are 54% and 87%, respectively

number of cases and percentage according to TNM staging (I/II/III/IV) were 15 (31.25%), 1 (2.083%), 29 (60.417%), and 3 (6.25%), respectively. The associations between Tregs and PRL, the tumor morphological features, and TNM staging were analyzed. Neither the frequency of Tregs nor the frequency of mTGF-beta on Tregs showed any association with morphological features or staging of HCC. However, multiple focal lesions were associated with significantly higher serum PRL levels (Table 2).

Correlation between frequency of Tregs and the studied clinical and laboratory data among HCC patients

The correlation of Tregs with studied clinical and laboratory data among HCC patients is presented

Table 2 Relationship between prolactin and the frequency of studied T regulatory cell subtypes and tumor clinic-pathological status in hepatocellular carcinoma patients

		Prolactin	$CD4^+CD25^+$ *Tregs*	$CD4^+CD25^+CD127^-$ *Tregs*	*mTGFβ1/* $CD4^+CD25^+$ *Tregs*	*mTGFβ1/* $CD4^+CD25^+CD127^-$ *Tregs*
Size of nodule(s)	< 5 cm	15 (12 – 60)	3.3 (1.7 – 6.3)	67 (52.3 – 82.1) (1)	23 (13.7 – 38.8)	16.3 (7.2 – 25.9)
	≥ 5 cm	15 (12 – 38)	5.8 (1.1 – 13.2)	59 (32 – 77.5)	31.8 (14.4 – 59.6)	15.2 (3.5 – 36.7)
	P-value	0.88	0.34	0.30	0.24	0.93
Number of nodule(s)	Solitary	12 (10 – 27)	3.4 (1 – 9.3) (1)	66.4 (35.8 – 83.9)	26.5 (12.1 – 64)	20 (6.1 – 32.7)
	Multiple	28 (15 – 50)	5.1 (1.1 – 9.4)	63.2 (33.3 – 75)	30 (19 – 43.7)	14 (4.7 – 25)
	P-value	**0.02**	0.84	0.68	0.88	0.42
Portal vein thrombosis	Present	15 (12 – 52)	5.8 (1.8 – 9.6)	67.2 (44.2 – 84.7)	27.8 (13.6 – 54)	12.2 (5.3 – 25.9)
	Absent	15 (12 – 34)	2.7 (0.83 – 7.7)	57.2 (30.5 – 75.6)	29.9 (14.6 – 57)	19 (7.6 – 33.3)
	P-value	0.94	0.17	0.17	0.67	0.30

The data are expresses as median and 25–75% interquartile and compared by the Mann–Whitney test

Table 3 The correlation between the frequency of studied T regulatory cell subtypes and clinical and laboratory data

Variables	$CD4^+CD25^+$Treg				$CD4^+CD25^+CD127^-$ Tregs			
	Frequency		Expressed mTGFβ_1		Frequency		Expressed mTGFβ_1	
	r	*P*	*r*	*P*	*r*	*P*	*R*	*P*
Age (years)	0.12	0.40	−0.06	0.674	−0.01	0.988	−0.093	0.531
hemoglobin (gm/dl)	0.56	**0.001**	0.33	**0.024**	−0.11	0.437	0.446	**0.001**
White blood cells × 10^3	−0.08	0.58	0.07	0.648	0.01	0.949	0.137	0.354
Platelets × 10^3	0.01	0.94	−0.06	0.695	−0.13	0.379	0.047	0.749
Alanine aminotransferase (U/l)	0.03	0.81	0.30	**0.038**	−0.14	0.350	0.361	**0.012**
Aspartate aminotransferase (U/l)	−0.13	0.37	0.02	0.897	−0.17	0.251	0.181	0.218
Total billirubin (mg/dl)	−0.16	0.26	0.08	0.588	0.20	0.166	0.236	0.106
Albumin (gm/dl)	0.39	**0.006**	0.18	0.055	−0.28	0.055	0.111	0.453
International normalized ratio	−0.083	0.57	0.06	0.665	0.005	0.971	0.137	0.353
Serum prolactin (ng/ml)	−0.03	0.80	−0.21	0.16	0.08	0.58	−0.30	**0.03**

CD cluster differentiation, *Tregs* T regulatory cells, *mTGFβ*$_1$ membrane-bound transforming growth factor β_1

Correlations were performed using non-parametric Spearman test. Bold values indicate statistically significant result

in Table 3. The traditional Treg (CD4 + CD25 +) frequency was positively correlated with hemoglobin and serum albumin levels. The frequency of expressed mTGF1 on both Tregs had significantly positive correlations with hemoglobin and ALT enzymes, and the frequency of expressed mTGF1 on CD4 + CD25 + CD127- Tregs had significantly negative correlations with serum PRL level (Fig. 3).

Discussion

To the best of our knowledge, our study is the first to address the frequency of peripheral blood CD4 + CD25 + CD127-Tregs and identify the expression of mTGF-ß1 on these Tregs as a marker for their immunosuppressive function, and we also were pioneers in assessing the interaction of serum PRL with these Tregs among HCV-related HCC patients. We demonstrated

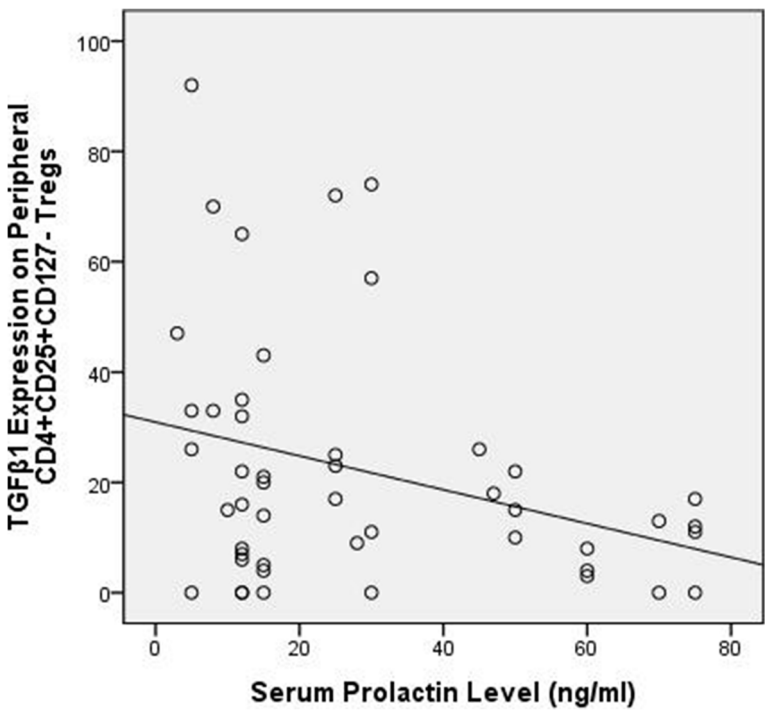

Fig. 3 The correlation between the frequency of mTGF-ß1 on CD4 + CD25 + CD127- Tregs and serum prolactin ($r = -0.30$, $p = 0.03$)

the higher immunosuppressive function of Tregs and higher PRL levels in HCC and cirrhotic patients than in healthy subjects, in spite of the similar frequency of these Tregs in the three groups and an insignificant difference between cirrhotic and HCC patients with regard to the immunosuppressive function of Tregs as well as serum PRL levels.

According to our findings, mTGF-ß1 on Tregs is a useful circulating specific and non-sensitive biomarker for HCV-related HCC diagnosis in healthy subjects but not in HCV-related cirrhotic patients. Similar to our results regarding traditional Tregs and CD4 + CD25 + CD127-Tregs with mTGF-ß1 expression have previously been reported in patients with HCC of various etiologies versus healthy subjects, as well as in HBV-related HCC versus cirrhotic and healthy subjects [17, 20].

On the other hand, assessment of this phenotype of peripheral Tregs, CD4 + CD25 + CD127-Tregs, in HBV-related HCC, and in primary liver cancer showed unmatched results; Tregs were lower in HBV-related HCC and higher in primary liver cancer compared to healthy subjects. After surgical resection of HCC, the level of Tregs increased in HBV-HCC while it decreased in primary liver cancer [19, 37]. There have been few studies that have looked at other subtypes of Tregs in HCV-related HCC. One study showed that peripheral CD4 + CD25 + Foxp3 + Tregs were higher in cirrhosis and HCC patients than healthy subjects, with a similar frequency in diseased groups [38]. Moreover, another study reported not only elevated peripheral Tregs but also the frequency of Tregs was positively correlated to viral load Egyptian cirrhotic and HCC patients with HCV [39]. Another tow studies looked at traditional CD4 + CD25 + Tregs in a small number of HCC patients with different etiologies, including HCV. Traditional Tregs were much more prevalent in the HCC group compared to the healthy controls, HCV-related cirrhosis, and chronic hepatitis C groups in the first study. There was also a similar frequency of Tregs in the two latter groups. The second study found that the frequency of Tregs increased gradually from healthy subjects to cirrhotic patients to HCC patients [40, 41].

However, the CD4 + CD25 + CD127- Tregs population is the most standard characteristic of Tregs. Concurrently, Tregs had a significantly higher expression level of the intracellular Foxp3 protein than other cell populations. However, labeling of CD4 + CD25 + CD127- on the cell surface is more precise and more representative for detecting Tregs than the labeling of CD4 + CD25 + Foxp3 + [30].

Our results regarding higher peripheral Tregs in HCV-cirrhotic patients than healthy subjects were matched with previously reported findings [38, 39]. In addition, CHC-infected Egyptian patients displayed high serum levels of Treg-associated cytokines [42]. Our finding and previous studies supported the hypothesis that liver damage in HCV-cirrhotic patients might be due to an immune-mediated destructive mechanism. Of note, the frequency of peripheral CD4 + CD25 + FOXP3 + Tregs was reduced in one study while increased frequency of Tregs was reported in another study among cirrhotic patients with hepatitis B [43, 44].

In our study, TGF1 expression on peripheral CD4 + CD25 + CD127-Tregs was matched in HCC and cirrhotic patients. The following points may help explain this finding: to begin, one hypothesis advanced is that peripheral Tregs are recruited to the local HCC environment in response to released chemokines because the frequency and suppressive function of Tregs infiltrating HCC tumors are greater than those in peripheral blood [45, 46]. Additionally, the persistence of suppressive activity of Tregs may lead to immune evasion in HCC although the relative proportions of Tregs may remain unaltered in HCC and cirrhotic patients [47]. Altered TGF-β signaling rather than expressed TGF-β levels may lead to tumor outgrowth and progression [48]. Finally, the apparent dual behavior of TGFβ as both pro- and anti-tumorigenic cytokines may add a further level of complexity to the mechanisms that regulate the interactions among cancerous, stromal, and immune cells within HCC. TGFβ can affect the transendothelial recurtement and migration of Tregs to tumor via counteracting mechanisms: it may attract Tegs directly and can also counteract the accumulation of intratumor Tregs. So, better manipulation of the TGFβ signaling may provide a therapeutic target in HCC patients [49]. Tregs exert their suppressive function via several mechanisms; membrane-bound TGF-β1 is particularly important in the suppression of effector T cells. TGF-β1 produced by inducible Tregs can induce other naïve CD4 + CD25 + cells to become similar suppressor cells. So, Tregs can secrete and respond to TGFβ in an autocrine manner. Also, TGF-β1is a central regulator in chronic liver disease contributing to all stages of disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and HCC. In addition, TGF-β1 has an important role in organizing a favorable microenvironment for tumor cell growth and enhancing epithelial-mesenchymal transition [16, 49].

In the current study, no association was found between frequencies of Tregs or mTGFβ1 expression and clinicopathological features and staging of HCC. These findings were in agreement with the meta-analysis study of Zhao et al. [18]. However, contradictory results were also described by others: both positive and negative associations between Tregs frequency and tumor size, tumor

vascular invasion, and HCC staging [19, 50]. Ongoing clinical trials aim to deplete Tregs or block TGFβ pathway in solid tumors, including HCC [51, 52].

The cross-talk between erythropoietin (EPO) and TGF- can explain the association between haemoglobin level and Tregs in our study. Unfortunately, EPO was not assessed in our study. The TGF-1 pathway (Smad3/4) in this context stabilizes the transcription factors mediating the hypoxia complex that controls EPO expression [53]. Furthermore, EPO treatment increases TGF- expression and activation in a human hepatoma cell line (Huh-7 cells); in the opposite direction, TGF- treatment significantly increased EPO production in a human hepatoma 3B cell under hypoxic conditions [54]. In HCC, hypoxia has been implicated in metabolic reprogramming of both immune and tumor cells, preventing immune cells from eliciting their full antitumor activities while sustaining the growth and survival of tumor cells. Hypoxia induces significant recruitment and enrichment of Tregs within the tumor via chemokine genes [55, 56].

We were the first to find a link between serum albumin and CD4 + CD25 + Tregs in HCC patients, as well as between serum albumin and TGF-β1 expression on both traditional Tregs and CD4 + CD25 + CD127- Tregs. Previous research found that administering albumin significantly increased the percentage of Tregs and TGF-1 in ischemic stroke patients [57]. Furthermore, Samuel et al. (2014) observed that in HIV-infected patients, a lower CD4 T cell count was associated with lower hemoglobin and albumin concentrations [58].

Our findings revealed a significant positive correlation between TGF-β1/Tregs and ALT in HCC patients. Tregs suppression of the immune response leads to the persistence of viral infection, inflammation, and liver damage in HCC patients, in addition to the predominant inflammatory action of TGF-β1 [59]. Tregs and ALT showed variable associations in different study populations; a positive association was described in chronic hepatitis B and cirrhotic patients, and a negative association was reported in chronic hepatitis C and HCC. Other researchers failed to find any association between ALT and Tregs in chronic hepatitis C patients [38, 41, 60, 61]. Among chronic hepatitis C patients, the normal ALT subgroup had significantly greater CD4 + CD25 + Tregs than the raised ALT subgroup [60].

In our study, serum PRL levels were significantly higher in HCC and cirrhotic patients than in healthy subjects, with no difference between the diseased groups. PRL was also found to be positively related to multiple hepatic lesions. Authors presented unmatched data on PRL levels in HCV-related HCC on top of liver cirrhosis in Egypt; Compared to cirrhotic patients, patients with HCC and healthy subjects had significantly lower PRL levels along with an insignificant difference between HCC and healthy subjects in one study [31]. Contrarily, others reported higher serum PRL levels in HCC patients than in cirrhotic and healthy subjects. Moreover, they identified a serum PRL level ≥ 44.5 ng/ml as a diagnostic cut-off value for HCC. The latter study's results, however, are complicated by the severity of liver dysfunction in HCC patients [32].

Also, patients in the Child A class with HCC of various underlying etiologies had significantly higher PRL levels than chronic hepatitis B patients [29]. In fact, elevated PRL in cirrhotic patients (not in chronic hepatitis) is correlated with the severity of liver dysfunction [62]. Impaired hepatic metabolism of estrogen, ineffective elimination of hormones, and disturbed secretion and feedback mechanisms are contributors to elevated PRL levels in cirrhotic patients [63].

Elevated PRL levels have also been reported in other types of cancer. It may be useful as part of multi-marker diagnostic panels, but not as an independent cancer biomarker, including HCC. It acts as an autocrine and paracrine growth factor, promoting the development and progression of carcinomas such as lung and ovarian cancer [64, 65]. In HCC tissue, increased mRNA and protein expression, as well as PRL gene amplification, were observed [66]. PRL has been described as having both oncogenic and anti-oncogenic effects in HCC. PRL may promote HCC progression by directly and indirectly activating the Janus kinase-STAT3 pathway [28, 66]. However, a controversial study found that it protects mice from HCC [27]. The differences between the studies may be explained by different types of PRL receptor isoforms. Binding of PRL to the short-arm PRL receptor, which is primarily expressed in normal liver tissue, inhibits hepatocarcinogenesis, whereas binding to the long-form arm PRL receptor (LFPRLR), which is primarily expressed in HCC tissues, may promote HCC progression through the activation of several pathways [27].

A novel finding of our study was that the suppressive function of Tregs was negatively correlated with serum PRL among HCC patients. In accordance with this finding, PRL physiologically inhibits the suppressor effect of peripheral Tregs in healthy subjects as Tregs are constitutively expressed PRL receptors [23]. However, binding of elevated serum PRL to LFPRLR on tumor parenchyma augments local recruitment of Tregs to malignant tissue via an increased chemoattractant, CCL17, in breast cancer. Furthermore, this is linked to an increased risk of cancer progression. The knockdown of the PRL long receptor had no effect on function of peripheral Tregs [67].

We suggested that similar mechanisms may occur in HCC parenchymal cells that predominantly express the

long-arm prolactin receptor and subsequently enhance tumor progression. This matched a reported positive association of serum prolactin with multiple focal lesions. On the basis of our findings and previous data, we suggested that elevated serum prolactin may interact with peripheral Tregs and exert protective effects on HCC development via immunological mechanisms, including inhibition of the suppressive effect of Tregs independent of prolactin long receptors. However, elevated serum prolactin enhances HCC multiplicity via several mechanisms, including immunological pathways with increased local recruitment of Tregs to tumor tissue. Further studies are needed to explore and discuss our findings.

Conclusion

Cirrhotic patients with and without HCC had higher serum prolactin levels and mTGF1 expression on traditional (CD4+CD25+) and well-characterized CD4+CD25+CD127- Tregs (not the frequency of Tregs) than healthy subjects. We concluded that mTGF1 on Tregs could be used as a specific but insensitive biomarker for distinguishing HCV-related HCC from healthy subjects but not HCV-related liver cirrhosis. Tregs are not related to clinicopathological features or the staging of HCC. Serum PRL maintained its physiological function as an immunogenic role by inhibiting the suppressive function of peripheral Tregs. It also has an oncogenic role as it is correlated with multiple focal lesions of HCC. TGF-β1 on peripheral Tregs was associated with HB levels, hepatic inflammation, and liver damage. We suggested further study to assess the interaction of circulating prolactin with local Tregs and parenchymal tumor tissue of HCC and measure erythropoietin to understand the cross-talk between Hb levels and Tregs. Our study's limitations were that a cross-sectional study cannot establish a causal relationship, it is small, and it is monocentric. We did not measure Tregs infiltrating tumor tissues.

Abbreviations

HCC	Hepatocellular carcinoma
HCV	Hepatitis c virus
Tregs	T regulatory cells
TGF-ß1	Transforming growth factor-β1
mTGF-ß1	Membranous–bound TGF-ß1
HBV	Hepatitis B virus
PRL	Prolactin
EASL	European Association for the Study of the Liver
TNM	Tumor node metastasis
AJCC	The American Joint Committee on Cancer
HIV	Human immune deficiency virus
ELISA	Enzyme-linked immunosorbent assay
PBMCs	Peripheral blood mononuclear cells
ALT	Alanine transaminase
EPO	Erythropoietin
LFPRLR	Long-form prolactin receptor
ROC	Receiver operating characteristic

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Not applicable.

Authors' contributions
Prof. MAA and Dr. RAM equally contributed to the conception and design of the research and equally contributed to the interpretation of the data; and RAM drafted the manuscript. MAA was responsible for patients' inclusion. Prof. DMS was responsible for flow cytometric analysis of the study. All authors critically revised the manuscript, agreed to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

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Availability of data and materials
The datasets used in this study are available from the corresponding author on rational request.

Declarations

Ethics approval and consent to participate
This study was carried out after approval of the local ethical committee of the Faculty of Medicine, Minia University. This was in accordance with the ethical standards and with the Helsinki Declaration of 1975. Written informed consent was obtained from each participant in the study.

Consent for publication
All the authors involved in this study give their consent for this article to be published in ***The Egyptian Liver Journal***. Not applicable for study subjects.

Competing interests
The authors declare that they have no competing interests.

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