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Evaluation of $CD4^+CD25^+$ regulatory T cells in patients with hepatocellular carcinoma and liver cirrhosis

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Abstract

The emergence of a Tumor results from the disruption of cell growth regulation as well as from failure of the host to provoke a sufficient immunological anti-tumor response. Regulatory T cells CD4+CD25+ (Tregs) play an important role in maintaining peripheral self-tolerance, thus preventing autoimmune pathologies. However, in certain situations Tregs can impair effective immunity to some pathogens and tumor cells. Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death in the world, and in developed countries it is expected to continue to increase because of the epidemic of chronic hepatitis C virus (HCV) infection. Previous studies also showed that Tregs infiltrating HCC tumors were an indicator of poor prognosis.

Aim: of this study was to evaluate CD4+CD25+ Treg cells in patients with HCC and liver cirrhosis and their correlation with liver tumor markers and grading.

Patients and Methods: The study included 30 patients, 15 patients had HCC (group I) and 15 were cirrhotic patients (group II). In addition, 10 healthy subjects were used as controls. All patients were subjected to clinical examination and laboratory investigations including liver function tests, hepatitis B markers (HBs Ag, HBeAg and HBc-Ab) and HCV antibodies were detected by ELISA. and Bilharzial Abs by indirect hemagglutination test. $CD4^+CD25^+$ Tcells were quantified in the blood by flow cytometry, alpha feto protein by Cobas e 411, To evaluate HCC grading ,abdominal sonography, C.T.and liver biopsy were done. Patients were categorized into mildely differentiated (grad 1), moderately differentiated (grad 11) and poorly differentiated (grad 111).

Results: There were significant increased in serum AFP, and $CD4^+CD25^+$% in patients with HCC, and in patients with liver cirrhosis when compared to control group ($p<0.05$), and highly significant increased in AFP, and CD4+CD25+ % in patients with HCC when compared to patients with liver cirrhosis ($p<0.001$). In HCC patients there were 2 patients (13.3%) of grade I, 10 patients (66.7%) of grade II and 3 patients (20%)of grade III .Regression analysis showed negative significant correlation between $CD4^+CD25^+$% and ALT ($p<0.05$, $r=-0.51$), and positive significant correlation between CD4+CD25+ and AFP ($p<0.05$, $r=0.41$) among patients with HCC. Also there was positive correlation between CD4+CD25+ and ALT ($p<0.05$, $r=0.46$) among patients with liver cirrhosis.

Conclusion: Our finding suggests that increased frequency of Treg cells might play a role in modulation of the immune response in HCC and liver cirrhosis, through limitation of the efficacy of anti-tumor response. Treg cells correlate properly with AFP and with tumor grades; this may play a major role in the inflammatory activity in the liver. Better understanding of the underlying mechanism of Tregs regulation may help to find immunotherapy for HCC and enhancing immunity against cancer.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumour over the world **(Guido et al ; 2004 and Lovet et al; 2003)**.
AFP is the most established tumour marker in HCC and the gold standard by which other markers for the disease are judged **(Guido et al; 2004)** the first serologic assay for detection and clinical follow-up of patients with HCC was measurement of AFP, allowed sequential studies in high risk patients and patients being treated with either surgical resection or chemotherapy **(Bartlett et al; 2005)**.
Studies have shown that biological behaviors such as metastasis of HCC are associated with a unique immune response signature of the liver microenvironment **(Budhu et al; 2006),** where lymphocytes play an important role through immunity and inflammation. Moreover, tumor progression in spite of the presence of substantial lymphocytic infiltration **(Harlin et al; 2006)** implies that the tumor microenvironment inactivates anti-tumor effectors T cells or induces immune tolerance **(Zheng et al; 2008)**.
CD4+CD25+ regulatory T cells (Tregs) are important in maintaining self-tolerance and regulating immune responses in both physiologic and disease statuses**(Ormandy et al; 2005).** Many studies have revealed that Tregs play a potential role in the pathogenesis of a variety of digestive system diseases, including autoimmune liver diseases (**Jones et al; 2002)** chronic hepatitis C and B **(Xu et al; 2006),** and gastrointestinal cancers **(Zheng et al; 2008).**
Studies have suggested that Tregs have a positive effect on tumor progression through suppression of effective anti-tumor immunity **(Gallimore and Sakaguchi, 2002)**, and removal of CD4 + CD25 + Tregs restores the immune response to tumors in vivo **(Jones et al; 2002 and Lovet, 2003).** Tregs were increased in peripheral blood (PB) and/or tumor in situ in HCC patients **(Yang et al; 2006)** and that increased Tregs suppressed CD4 helper T-cell responses and appeared to promote HCC progression**(FU et al; 2007).**
Both hepatitis B virus (HBV) and hepatitis C virus (HCV) can cause persistent viral infection in humans. Chronic infection is associated with a risk of cirrhosis and hepatocellular carcinoma. A large body of evidence suggests that a failure of the adaptive immune response is critical in the establishment of chronic infection **(Simon et al; 2007).**
An abundance of experimental data has confirmed that CD4 + CD25 + T regs may play an important role in the suppression of virus-specific immunity. In particular, in chronic infections caused by HBV and HCV, the frequency and functional properties of CD4+CD25+ Tregs may contribute to chronic virus development and influence the course of the disease by suppressing antiviral immunity **(Zheng et al; 2008)**.
HCC in patients with chronic viral hepatitis is of major clinical importance, especially as therapy for HCC is so poor. The major risk factors for HCC are the presence of cirrhosis and male gender. However, the risk of HCC is higher in HBV and HCV infection than for other forms of cirrhosis; it is unknown whether the accumulation of intrahepatic T-regulatory cells increases the risk of malignancy by inhibiting antitumor responses but it is clear that HCC in patients with chronic viral hepatitis is infiltrated by T-regulatory cells **(FU et al; 2007).**
The aim of this study was to evaluate CD4+CD25+ Treg cells in patients with HCC and liver cirrhosis and their correlation with liver tumor markers and grading.

Patients and Methods

The present work was carried out on 40 subjects attending to internal medicine, hepatology and gastroenterology departments, Al-Zahrra university hospital, and Surgical gastrointestinal unit of Benha teaching hospital, during the period from April 2009 to December 2010.
Subjects were divided into 3 groups:
Group I: include15 patients with HCC, 9/15 (60%) of them were males and 6/15 (40%) were females. Their age ranged from 42-70 years (mean 53.8 ±7.6). 66.7% (10/15)of them were suffered from hepatitis C, 13.3% (2/15) hepatitis B and 20% (3/15) bilharziasis.
The diagnosis of HCC cases was done by : abdominal sonography, abdominal triphasic C.T.

and typical hsitopathological findings of focal lesion in the liver . The lesions were of grade I histopathologically in 2 patients (13.3%), grade II 10 patients (66.7%) and grade III 3 patients (20%). Histological grading were performed using a modified Knodell scoring system by a pathologist blinded to the clinical ***data*** **(Ishak et al; 1995)** .

Group II: Include15 patients with liver cirrhosis, diagnosed by clinical, laboratory, liver biopsy guided by U/S and surgical abdominal labroscopy under vesion to avoid bleeding from focal lesion of liver (HCC), whenever possible. 10/15 (66.6%) of them were males and 5/15 (33.3%) were females. Their age ranged from (40-65 years) and their mean (55.9 ±9.3). 73.4% of them were suffered from hepatitis C (11/15), 13.3% hepatitis B (2/15) and 13.3% bilharziasis (2/15).

Group III: Control group: 10 healthy control subject 5/10 (50%) of them were males and 5/10 (50%) of them were females. Their age ranged from (44-58years) with mean of (52.9±6.2). They had normal values of serum alanine aminotransferase (ALT) and were seronegative for hepatitis B markers (HBs Ag,HBeAg and HBc-Ab), HCV and bilharziasis.

Patients suffering from renal failure, cardiac failure and carcinoma elsewhere were excluded from the study. No antiviral therapy during the 6 months before blood sampling.

Patient Samples :

Blood samples were obtained by peripheral venipuncture from patients with minimal stasis after informed consent and aseptic conditions. Ten ml of blood were withdrawn from each case. 2 ml of whole blood were mixed with EDTA to perform CBC and CD4+CD25+ (Treg) cells, 1.8 ml of blood were mixed with 200 µL sodium citrate to perform prothrombin time. The remaining blood samples were taken in plain tube, put in water path at 37 C° for 30 minutes and centrifugation was done for 10 minutes , the resultant serum was divided in a liquots for measurements of (alph feto protein, clinical chemistry tests and serological tests).

All patients and controls were submitted to the following:

1. Full history and physical examination.
2. Routine laboratory investigations including :

- Complete blood counts (CBC) using fully automated cell counter (Sysmex Kx-21-Japan)
- liver function testes (AST, ALT, protein, albumine, total Bil., and ALP) kidney function testes (blood urea, serum creatinine) all of them were measured on Hitachi 911 autoanalyzer using Rouche reagent kits.
- Prothrombin time, concentration and INR were done by coagulometer (Sysmeix CA-500).
- Seological test: HBsAg, HBeAg and HCV Abs by ELISA technique using (SLT. SPECTRA II A-5082 AUSTRIA) reader and kits of DIA. PRO (Diagnostic Bioprobes Milano Italy). Bilharzial Abs by indirect hemagglutination test using Siemens reagent kit.
- Tumor markers: Alph feto protein by Cobas e411 (immuno- chemiluminescent) autoanalyzer using Roche reagent kits.
- Treg cells CD4+CD25+: by flowcytometry. Frequency of $CD4^+$ $CD25^+$ Tregs by flowcytometric analysis using the human Treg cell staining kit (eBioscience)(BD Biosciences) Data acquisition and analysis were performed on Coulter EP-ICS XL flow- cytometry. Lymphocytes were gated via their forward and side scatter properties. To determine the Treg cell 100ul of whole blood sample added to 1ml isotone to make count 5000-1000, 20 uL of diluted sample was mixed well with the appropriate monoclonal antibodies (antiCD25-Phycoethrin), (anti CD4-Fluroscein isothiocyanate) (Beckman Coulter,USA) and incubated for 15 minute, after that one ml of lysing reagent was added ,vortex was done and the sample was reading within one minute. Treg cells was electronically selected on the basis of their side and forwared scatter characteristics and 10000 cells were analyzed in each sample.

Statistical analysis: The statistical analysis of data was done by using statistical package for social science (SPSS) version 16 on windows XP.

The description of data was done as frequency and proportion for qualitative data, mean ± SD for quantitative data. The analysis of data was done to test statistical significant difference for quantitative data using student's t test. For qualitative data [frequency & proportion] chi-

square test was used. Measuring the mutual correspondence between two values was done using the pearson's correlation coefficient. P value was considered significant when it is ≤ 0.05.

Results

Baseline clinical and laboratory characteristic of the studied groups is provided in table (1). There was significant increased in AFP, in patients with HCC, and in patients with liver cirrhosis when compared to control group ($p<0.05$), and highly significant increased in AFP, in patients with HCC when compared to patients with liver cirrhosis $p<0.001$ table (2). Also there was significant increased in $CD4^{+}CD25^{+}$ % patients with HCC, and in patients with liver cirrhosis when compared to control group ($p<0.05$), and highly significant increased in CD4+CD25+, in patients with HCC when compared to patients with liver cirrhosis $p<0.001$ fig (1). In HCC patients there were 2 patients (13.3%) of grade I, 10 patients (66.7%) of grade II in and 3 patients (20%)of grade III

Table (3) fig (2) show correlation between CD4+CD25+ and each albumin, ALT, INR, and AFP in group I (patients with HCC) There were negative significant correlation between CD4+CD25 and ALT ($p<0.05$, $r=-0.51$) and positive significant correlation between CD4+CD25+ and AFP ($p<0.05$, $r=0.41$). Among group II (patients with liver cirrhosis) positive there was correlation between CD4+CD25+, and ALT ($p<0.05$, $r=0.46$) . fig (3) represent lymphocyte gating using forword scatter versus side scatter. $CD4^{+}$ cells were acquired after gating the lymphocyte population by forward- and side-scattered properties. and fig (4) represent data of dot plots for flow cytometry and the gating strategy of HCC, LC and control respectively(Gating approach for discrimination of $CD25^{+}$ cells). ***transonographic features of the studied patients*** revealed that hepatomegaly in 21.9% of HCC cases compared to 19.2% of cirrhotic patients, shrunken liver was present in 41.5% and 76.9% of HCC and cirrhotic patients respectively and was statistically significant. The coarse texture was present in 100% in the two groups. Portal vein thrombosis (PVT) was only present in HCC cases compared to the cirrhotic patients.

Table (1): *Clinical and laboratory data of the studied groups*

Parameter	Group I patients with HCC N=15 mean±SD	Group II patients with liver cirrhosis N=15 mean±SD	Group III control N=10 mean±SD
Age (years)	53.8±7.6	55.9±9.3	52.9±6.2
Sex (m/f)	9/6	10/5	5/5
Albumin (g/dl)	3.3±1.2	3.6±0.6	4.2±0.3
S.bilirubin (mg/dl)	1.7±1.2	1.8±0.7	0.83±0.14
Creatinin (mg/dl)	0.86±0.21	0.92±0.17	1.02±0.19
AST(U/L)	96.2±26.9	70.6±8.7	26.7±5.5
ALT (U/L)	59.1±18.9	60.8±9.5	24.6±5.9
INR	1.5±0.19	1.5±0.2	1.1±0.08
AFP ng/ml	1033.7±441.7	25.6±14.9	3.6±2.7
CD4+CD25+%	6.76±2.3	2.8±1.2	1.2±0.7
HBV (+v/-ve)	2/15	2/15	----
HCV(+v/-ve)	10/15	11/15	----
Bilharziasis(+v/-ve)	3/15	2/15	----

Table (2): *Comparison of mean value level of blood alpha feto protein and CD4+CD25+ in different studied groups*

parameter	Group I mean±SD	Group II mean±SD	Group III mean±SD	Group I vsGroupIII (Pvalue)	Group II vsGroupIII (Pvalue)	Group I vsGroupII (Pvalue)
AFP ng/ml	1033.7±441.7	25.6±14.9	3.6±2.7	<0.05	<0.05	<0.001
CD4+CD25+%	6.76±2.3	2.8±1.2	1.2±0.7	<0.05	<0.05	<0.001

Table (3): *Correlation between serum CD4+CD25+ and each of albumin, ALT, INR, and AFP in patients with HCC*

	parameter	R value	P value	significance
CD4+CD25+%	albumin(g/dl)	-0.05	>0.05	NS
	ALT(U/L)	-0.51	<0.05	S
	INR	0.13	>0.05	NS
	AFP ng/ml	0.41	<0.05	S

Table (4): *Correlation between serum CD4+CD25+ and each of albumin, ALT, INR, and AFP in patients with liver cirrhosis*

	parameter	R value	P value	significance
CD4+D25+%	albumin(g/dl)	0.04	>0.05	NS
	ALT(U/L)	0.46	<0.05	S
	INR	0.16	>0.05	NS
	AFP ng/ml	0.14	>0.05	NS

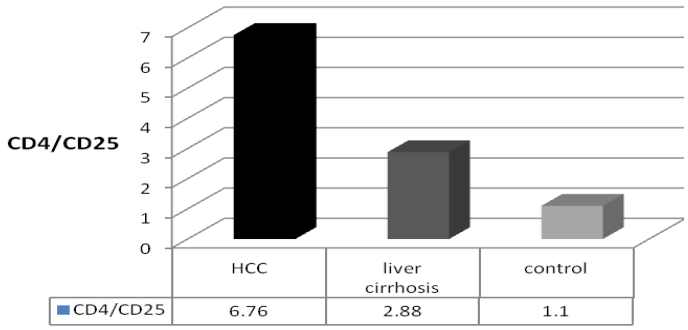

Fig (1): *Mean value of CD4+CD25+% among studied groups.*

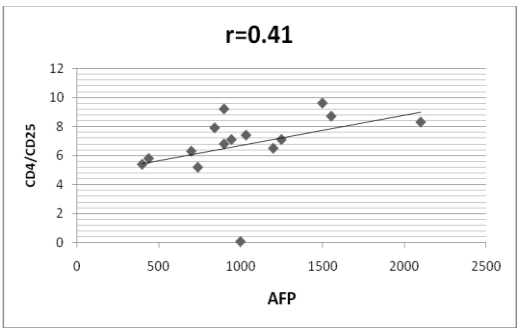

Fig (2): *Positive correlation between AFP and CD4+CD25+ among group I (Patients with HCC)*

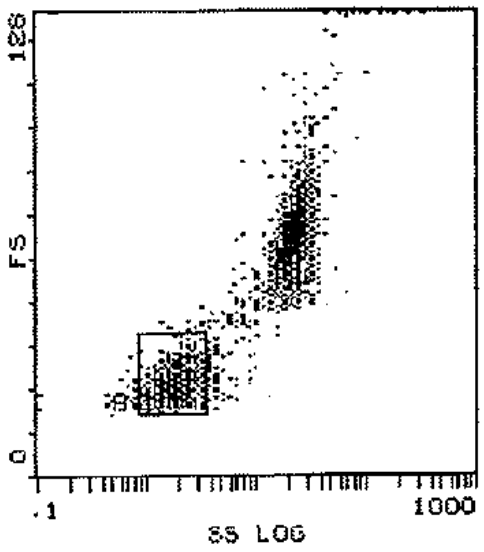

Fig. (3): *Lymphocyte gating using forword scatter versus side scatter*

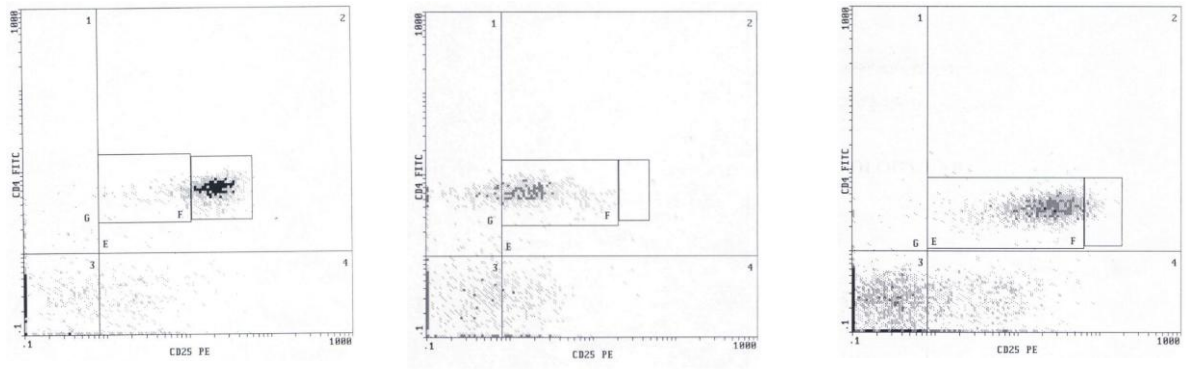

Figure (4): *Representative data of dot plots for flow cytometry and the gating strategy of HCC, LC and control respectively(Gating approach for discrimination of CD25^{+} cells).*

Discussion

The prognosis of HCC is generally grave **(Lopez , 2005**) approximately 75% of patients with hepatocellular carcinoma present with advanced, unresectable disease and some element of hepatic dysfunction **(Vauthey et al; 2002)** and in Egypt most patients presented in late stage in 85% of cases (**Abdel-Gafa et al; 2002)** .

In the present study HCC commonly presented in males more than females . This was in agreement with ***Di Bisceglie (2002)*** who reported that men are two to three times higher than women .

The same result was found in Egyptian series by ***Mohmad et al. (2000) and El-Zayadi et al.(2005)*** who showed the higher susceptibility of males to environmental carcinogenic factors and greater exposure to them.

Dysfunction of the host immune system in cancer patients can be due to a number of factors, including suppression of tumor-associated antigen reactive lymphocytes by CD4+CD25+ regulatory T (Treg) cells. Several studies suggest that Tregs are elevated in cancer patients and that depletion of Tregs may enhance the antitumor immunity of host **(Cao et al; 2007).**

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death in the world, and in developed countries it is expected to continue to increase because of the epidemic of chronic hepatitis C virus (HCV) infection. Most patients present with advanced disease with limited treatment options that are palliative. During the development of HCC, the tumour microenvironment has been shown to play a major role in promoting progression via a variety of immunological mechanisms **(Cabrera et al;2010).**

Treg cells may suppress immune surveillance of malignant tumors including hepatocellular carcinoma (HCC). Elimination of Treg cells leads to a more effective antitumor immune reaction and causes more efficient tumor rejection, especially in the early stage of tumor growth. These findings may implicate Treg cells in the development of HCV-related HCC, because HCC frequently develops in patients with HCV-positive advanced chronic hepatitis and cirrhosis. Furthermore, these results may also lead to therapeutic strategies for the manipulation of Treg cells for eradication of chronic HCV infection and subsequent development of HCC **(Yoshizawa et al; 2010).**

In the present study peripheral blood of alph feto protein and CD4+CD25+ were measured in 15 patients with liver cirrhosis, 15 patients with HCC in comparison to 10 healthy controls.

Our results showed highly significant increased in CD4+CD25+ level in patients with liver cirrhosis compared to control group ($P < 0.05$). Our results agree with ***Yohizawa et al.(2010)*** who reported that Treg cells were significantly increased in Liver cirrhosis ($P < 0.001$) compared to healthy control, but ***Lian et al., (2009)*** found that there were no differences between those with liver cirrhosis and controls ($p>0.05$).

Also our results showed highly significant increased in serum CD4+CD25+ level in patients with HCC when compared to control group ($P < 0.05$). These results are in accordance with ***Yohizawa et al. (2010)*** who reported that Treg cells were significantly increased in patients with HCC ($P<0.0001$) compared to healthy control. Also ***Cao et al. (2007)*** found that Treg cells were increased in peripheral blood from HCC patients. Also ***Shen et al. (2010)*** reported that the prevalence of Treg cells were significantly higher in the peripheral blood in HCC compared with those in normal donors.

Ormandy, et al. (2005) showed that, the frequency of CD4+CD25+ T cells in HCC patients were significantly higher in HCC patients than in healthy donors, and liver cirrhosis patients, and the prevalence of CD4+CD25+ cells in HCC patients were significantly higher ($P < 0.001$) than in healthy donors ($P < 0.01$).

The present study provided an additional insight into the regulatory mechanisms responsible for immunosuppression in human cancers, which facilitates local tumor growth and metastasis. Metastasis often represents the fatal step during the course of malignancy. Treg cells were correlated significantly with patients tumor grades(11 and 111) ($r=0.455, 0.510$). This suggest that tumor grades progression were enhanced by the suppression of immunosurveillance mechanisms.

The present study also show significant increased in CD4+CD25+ cells in patients with HCC compared to liver cirrhosis. These results in agreement with ***Yohizawa et al. (2010) and Cao et al. (2007)*** they reported that a significantly higher proportion of circulating CD4+CD25+ Treg cells were observed in HCV-related HCC when compared to healthy controls and HCV-related chronic liver dysfunction.

yang et al, (2006), and unitt et al. (2005) reported that circulating CD4+CD25+ Treg cells in HCC patients have not been increased compared to controls, or patients with CH or LC. On the other hand, those HCC patients had increased numbers of circulating Treg cells which were not correlated with the stage of the disease, several parameters contribute to these finding, including differences in patient profiles, disease stage and identification method of circulating Treg cells .

Our results showed a positive significant correlation between CD4+CD25+ and AFP, and a negative significant correlation between CD4+CD25+ and ALT among patients with HCC. Also, there was a positive significant correlation between CD4+CD25+ and ALT among patients with liver cirrhosis. Our data is in agreement with that of ***Zhou et al.(2010)*** who detectd that Tregs were associated with

AFP levels, and liver functions. Also a similar study by ***Sasaki et al. (2008)*** found a correlation between Treg and AFP.

Yohizawa et al. (2010) showed that the frequency of Treg cells in chronic hepatitis was not related to the grade of inflammation or the serum level of ALT, they hypothesized that the proportion of Treg cells may fluctuate in relation to the grade of inflammation of the liver.

Bolacchi et al. (2006) reported that CD4+CD25+ Treg cells were significantly greater in patients with normal ALT compared to patients with elevated ALT**.** In addition CD4+CD25+Treg cells from patients with normal ALT levels proved to be significantly more potent to suppress CD4+CD25+ Treg cells reactivity with respect to those from patients with elevated ALT.

Wolf et al.(2003) in their study mentioned that the depletion of Treg cells may become a successful anticancer strategy.

Conclusion: Treg cells correlate properly with AFP and tumor grades, these tumor specific CD4+CD25+Treg cells may limit the efficacy of anti-tumor response. A better understanding of the underlying mechanisms of Treg cells regulation in patients with HCC may allow a better diagnostic opportunities and give a chance for more effective and future immunotherapy.

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خلايا التى المنظمة (سى دى 4 و سى دى 25) فى مرضى كل من التليف الكبدى و سرطان الكبد و علاقتهم بدلالات اورام الكبد وتصنيفهم المرضى

أمل عبدالعليم مرسى, **ايمان عبدالرحمن وعبدالعاطى الغنيمى**
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يحتل سرطان الكبد المرتبة الخامسة في الأورام السرطانية على مستوى العالم. ويأتي معظم المرضى المصابين في مراحل متأخرة من المرض في حوالي 85% من الحالات، سيظل سرطان الكبد فى ازدياد فى الدول النامية بسبب التهاب الكبد الوبائى سى. وتلعب خلاياالتى المنظمة دورا مهما فى الحفاظ على المناعة الذاتية وتمنع حدوث الامراض المناعية, ولكنها فى بعض الاحيان تعمل على اتلاف تاثير المناعة لبعض الامراض وبعض الاورام. ويهدف البحث الى تقييم مستوى خلايا التى (سى دى 4 والسى دى 5) فى مرضى التليف الكبدى ومرضى سرطان الكبد و علاقتهم بدلالات اورام الكبد وتصنيفهم المرضى .

وقد تم إجراء هذه الدراسة على 15 حالة مصابة بسرطان الكبد، و15 حالة مصابة بالتليف الكبدى ، كما شملت الدراسة 15 فرداً آخرين أصحاء ظاهرياً دون وجود دليل على إصابتهم بمرض كبدي مزمن. تم فحص المرضى إكلينيكيا ومعمليا شاملاً صورة دم كاملة, وظائف كبد، دلالات فيروسات، نسبة الألفافيتوبروتين،خلايا التى (سى دى 4, سى دى 25) وأشعة بالموجات فوق الصوتية على البطن, كما تم التأكد من جميع حالات سرطان الكبد بالأشعة المقطعية أو العينة الكبدية في بعض الحالات. وقد تبين من هذه الدراسة أن معظم المرضى المصابين بسرطان الكبد كانوا من الرجال،الفحص الظاهرى للمرضى لم ينبأ عن وجود سرطان الكبد فيما عدا تضخم وتصلب الكبد،أثبتت الدراسة أن نسبة كبيرة من مرضانا لديهم إصابة بالالتهاب الكبدى الفيروسى (سى).معظم المرضى كانوا يعانون من الاعتلال الكبدى فى مراحله المتطورة. وقد أثبتت الدراسة بوجود فرق ذو دلالة إحصائية بين مسوى كل من نتيجة (سى دى 4 و سى دى 25) و اللألفافيتوبروتين لكل من مرضى سرطان الكبد ومرضى التليف الكبدى. وقد وجد أن هناك ارتباط ايجابي ذا دلالة إحصائية بين خلايا التى المنظمة (سى دى 4 و سى دى 25) و اللألفافيتوبروتين.

وقد استخلصت الدراسة: وجود علاقة ذات دلالة احصائية بين اللألفافيتوبروتين و خلايا التى المنظمة (سى دى 4 و سى دى25) وتصنيفهم المرضى ونخلص من ذلك بان العمل على الفهم الجيد لالية الخلايا المنظمة سوف يكون لة اثر جيد فى المستقبل للعلاج المناعى وتشخيص هذة الحالات المرضية.