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Value of Regulatory T Cell in Hepatocellular Carcinoma Patients

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, representing the second leading cause of cancer-related death worldwide**.** It is the 2nd leading cause of cancer death in men and the sixth among women.

Objectives: To investigate the role of CD4+, CD25+, FOXp3+Regulatory T cells (Tregs) in patients with HCC and their correlation with the prognosis.

Patients and Methods: The study was conducted on 25 patients with hepatocellular carcinoma. Detection of regulatory T cells by flow cytometry using CD4, CD25 and FOXp3 before and after intervention.

Results: CD4 significantly increased but CD4/CD25, Foxp3, AFP, ALT and AST significantly decreased. Patients with high numbers of FOXp3+ cells and those with higher FOXp3+ Tregs /CD4+ T cell ratio had poorer overall survival rates.

Conclusion: Tregs seem to be a promising prognostic factor in patients with HCC and a high Tregs level has been reported to be correlated with poor outcomes in a number of publications.

Keywords: HCC, CD4, FOXP3, CD25.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver. World Health Organization (WHO) considers HCC as the second leading cause of cancer deaths [1, 2, and 3]**.** HCC patients have an established background of chronic liver disease and cirrhosis, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alcoholic liver disease, and nonalcoholic steatohepatitis [3, 4]**.** Aberrant transformation of tumour cells and evolution of microenvironment are contributed to disease progression [5]**.**

The immune cells, in particular, regulatory T cells (Tregs), a subgroup of CD4+ T-helper cells, constitute a critical component in modulating local immune microenvironment and facilitates the evasion of tumor cell clearance by CD8+ cytotoxic lymphocytes (CTLs) [6, 7, 8]**.**

The transcription factor, forkhead box P3 (Foxp3), provides a biomarker for Tregs' detection, in addition to GITR+; LAG3+ and CD127low. Tregs mediate immunosuppression by secreting TGF-β1 and IL-10. The number of FOXp3+Tregs is markedly increased in both peripheral blood and tumor of HCC patients, which is linked to uncontrolled tumor growth and progression [9, 10]**.**

The aim of the present study was to investigate the role of CD4+, CD25+, FOXp3+Regulatory T-cells in patients with HCC and their correlation with the prognosis.

SUBJECT AND METHODS

This cohort study was carried out at Clinical Pathology Department and Advanced Hepato-Pancreatico-Biliary Centre in Zagazig University Hospital, in the period from March 2018 to September 2018. Twenty five subjects with HCC were included in this study. HCC was diagnosed on the basis of the results of alpha fetoprotein (AFP) determination and liver radiology techniques. The choice of patients based on the Barcelona Clinic Liver Cancer (BCLC) staging classification. All patients were at stage A (early stage). They were 7 females and 18 males with the age ranged from (40-76) in whom Treg detection was performed before and after the intervention.

Ethical approval:

An approval of the study was obtained from Zagazig University academic and ethical committee. Every patient signed an informed written consent for acceptance of the operation. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Inclusion Criteria: Age > 18 years, and hepatocellular carcinoma patients.

Exclusion Criteria: Patient's refusal, other malignancy, chemotherapy, and liver tumors other than HCC.

All patients included in this study were subjected to the following:

A- Routine laboratory investigations: after complete history taking, full clinical examination and abdominal ultrasonography, the following were done:

1- Measurement of T. bilirubin, D. bilirubin, total protein, S. albumin, ALT, AST, GGT and alkaline

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phosphatase, creatinine, bl. Urea and uric acid by cobas 8000 (Roche diagnostic, Japan).

2- Complete blood count (CBC) by Sysmex XN-2000 ((Siemens, Germany) in addition to prothrombin time (PT) and INR by using coagulation analyzer model CA1500 (Sysmex, Japan).

3- Viral markers including HBsAg and HCV-Ab, if positive. HCV-RNA using Realtime PCR.

4- Serum AFP level by cobas 8000 (Roche diagnostic, Japan).

B- Detection of regulatory T cells by using CD4, CD25 and FOXp3 by (FACS caliber 4 color Cell Quest Software", Becton Dickinson, San Joe, USA) using monoclonal antibodies on peripheral blood samples.

Principle of flow cytometry (FC):

Flow cytometry is a technique by which measurement of chemical or physical characteristics of cells is carried out as they move in flow in a fluid column and interrupt a beam of laser resulting in light scattering, permitting quantitative multi parametric measurement of cell properties. Antibodies specific for various cellular antigens can be labeled with different fluorochromes that can absorb and emit laser light, allowing FC analysis of two or more cell associated antigens. The cells are electronically gated using light scatter parameters (forward scatter reflecting the cell size, and the side scatter reflecting the internal structure of the cell) [(11)].

Monoclonal antibodies:

- ØAnti-Human: CD4 FITC conjugated: (supplied by eBioscience, catalog number: 110048, USA).
- ØAnti-Human: CD25 PE conjugated: (supplied by eBioscience catalog number: 120259, USA).
- ØAnti-Human: FOXp3APC conjugated: (supplied by eBioscience, catalog number: 174777, USA).

Specimen Collection:

Venous blood was aseptically collected, dispensed into 2 tubes of K-EDTA for CBC and flow cytometry for detection of CD4+ CD25+ FOXP3+Regulatory T cells (Tregs). The third Citrated tube used for PT and INR. The fourth one was a plain tube, used to collect serum to determine other investigations.

Statistical analysis

Data were collected, entered and analyzed by Statistical Package for the Social Sciences (SPSS version 20.0) software for analysis. According to the type of data, qualitative data were represented as number and percentage and quantitative continues group was represented as mean ± SD. the following tests were used to test differences for significance: differences between quantitative paired group by paired t, correlation by Pearson's correlation or Spearman's. P value was set at < 0.05 for significant results & < 0.001 for high significant result.

RESULTS

Table (1): Anthropometric, microbiologic, radiologic and interventional study

	Mean ± SD		**Median (Range)**
Age (years)	57.44 ± 9.96		60.0 (40-76)
Viral load	457656.71 ± 287102.4		379345.5 (100236-932557)
		N	**%**
Sex	Female	7	28.0
	Male	18	72.0
HCV	Negative	11	44.0
	Positive	14	56.0
Intervention	Microwave	5	20.0
	Radiofrequency array	5	20.0
	Resection	15	60.0
Focal lesion	Single	20	80.0
	Two	3	12.0
	Three	2	8.0
Focal lesion, size	Mean± SD	**5.02 ± 1.96**	

Table (2): Change assessment between pre and post intervention therapy among studied group

	Pre	Post	Paired t	P
HB (g/dL)	12.68±1.84	12.75±1.46	-0.660	0.516
WBCs (mcL)	6.28±1.11	6.26±1.77	0.066	0.948
PLT (mcL)	145.4±4.2	164.8±5.4	-5.573	0.00**
INR	1.16±0.22	1.2±0.31	-1.298	0.207
ALP (U/L)	173.08±6.8	172.32±8.6	1.088	0.287
Albumin (g/L)	3.76±0.59	3.77±0.6	0.165	0.871
TB (µmol/L)	1.9±0.09	1.78±0.08	0.582	0.566
ALT (U/L)	53.37±6.7	30.75±1.5	9.037	0.00**
AST (U/L)	52.8±4.14	30.45±1.88	7.487	0.00**
CD4	3.77±0.21	7.43±1.87	-5.513	0.00**
CD4/CD25	6.16±1.81	2.91±0.17	11.125	0.00**
Fox p 3	12.39±2.6	11.08±2.9	7.341	0.00**
AFP (ng/mL)	129.97±3.44	7.6±1.08	7.081	0.00**

This table showed that PLT and CD4 were significantly increased post intervention therapy, while CD4/CD25, Foxp3, AFP, ALT and AST are significantly decreased.

Table (4): Correlations between AFP, ALT, AST, with CD4, CD4/CD25, FOXp3 and Focal size

			AFP		ALT		AST	
			Pre	Post	Pre	Post	Pre	Post
CD4	Pre	r	.004	.179	-.080	-.217	-.345	-.338
		P	.985	.451	.704	.358	.091	.145
	Post	r	.176	-.018	-.324	.115	.064	.077
		P	.457	.940	.164	.630	.789	.748
CD4/CD25	Pre	r	.256	.130	.194	.238	-.072	.096
		P	.217	.586	.352	.313	.732	.688
	Post	r	.285	.129	.188	.272	-.147	-.132
		P	.223	.587	.428	.247	.537	.580
Foxp3	Pre	r	.036	.111	-.030	-.147	-.059	.193
		P	.864	.643	.885	.536	.779	.415
	Post	r	.120	.143	.066	-.143	-.013	-.008
		P	.613	.548	.784	.547	.957	.973
Focal size		r	.110	.407	.063	-.444	-.164	-.098

There was significant positive correlation between size and CD4 and negative between size and CD4/CD5.

Table (5): Univariate and multivariate analysis of factors associated with overall survival

Variables	Univariate		Multivariate	
	P-value	HR	95% CI	P-value
AFP (ng/ml)	0.4931	NA	NA	—
HCV: positive/negative	0.9413	NA	NA	—
Tumor size	0.4327	NA	NA	—
Tumor number: single/multiple	0.0261*****	NA	NA	—
FoxP3$^+$ Tregs %	0.0053*****	NA	NA	—
CD4+/CD25hiegh	0.0051*****	3.631	1.541–	0.002*****
FoxP3$^+$ Tregs/CD4$^+$ T cells ratio	0.0053*****	3.641	1.608–8.451	0.001*****

Cox proportional hazards regression model. HR = Hazard Ratio. NA = not adopted.

This table showed that, the patients with more than one tumor had worse prognosis (P = 0.0261). Investigation for the association with overall survival by using the log-rank test showed that patients with high numbers of FOXp3+ cells and those with higher FOXp3+ Tregs /CD4+ T cells ratio had poorer overall survival rates.

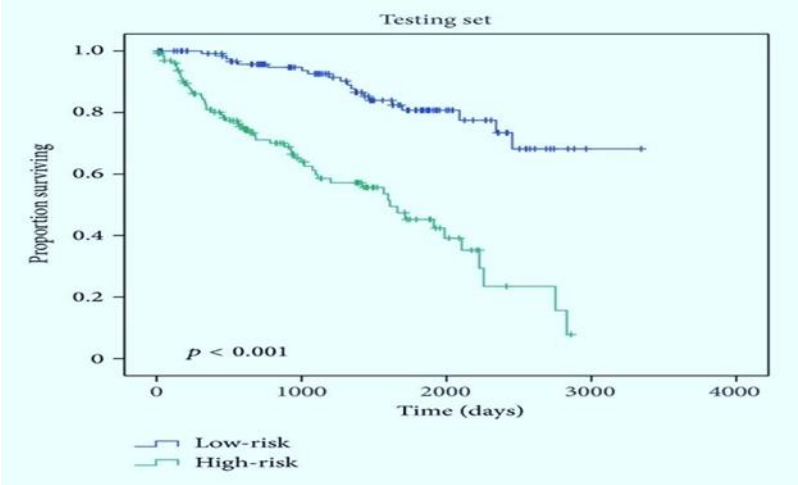


Figure (1): Kaplan-Meier survival curves using the log-rank test to further investigate their association with OS.

Table (6): A receiver operating characteristic (ROC) curve for FoxP3+ Tregs/CD4+ T cells ratio and FoxP3+Tregs/CD25 T cells ratio

Test Result Variable(s)	Cutoffs	P	95% Confidence Interval		Sensitivity	Specificity
			Lower Bound	Upper Bound		
Tregs.	3.1	0.01**	0.983	1.000	75%	67%
FoxP3+Tregs/CD4+ T cells	0.31	0.02**	0.872	1.000	86%	62%
ratio.	0.34	0.03	0.761	1.000	62%	67%

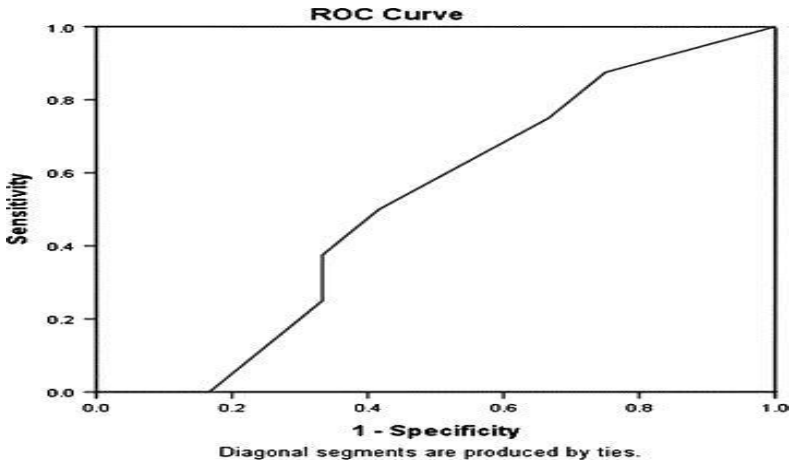
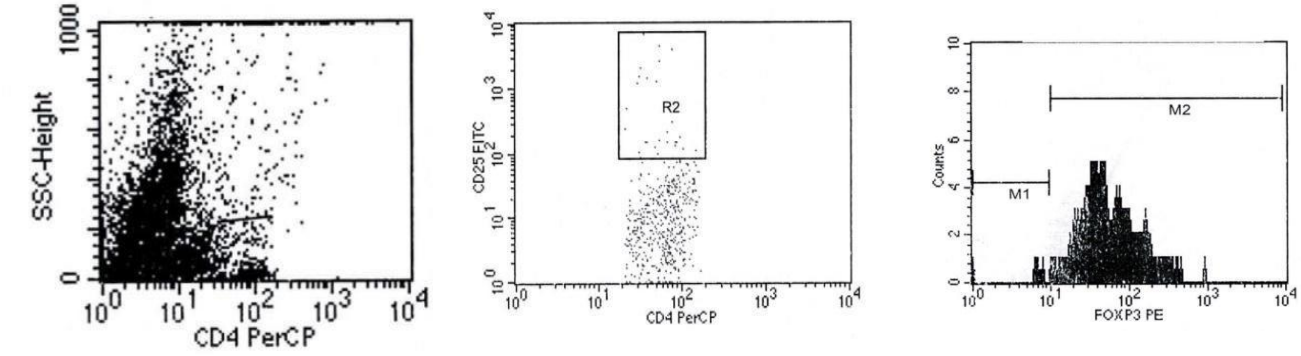


Figure (2): A receiver operating characteristic (ROC) curve for FoxP3+ Tregs/CD4+ T cells ratio and FoxP3+ Tregs/CD25 T cells ratio.

PBMCS from HCC patients before & after treatment were fixed & stained with anti CD4, CD25 & FOXP3, gating strategies showed population of lymphocytes, which had been selected for detection of CD4 cell to exclude the non-lymphocyte gating on lymphoid cells based on forward side scatter characteristics **(A).**



R2 is the population of Treg. Histogram plots for FOXP3 on lymphocyte CD4, CD25. CD4, CD25 population **(B)**

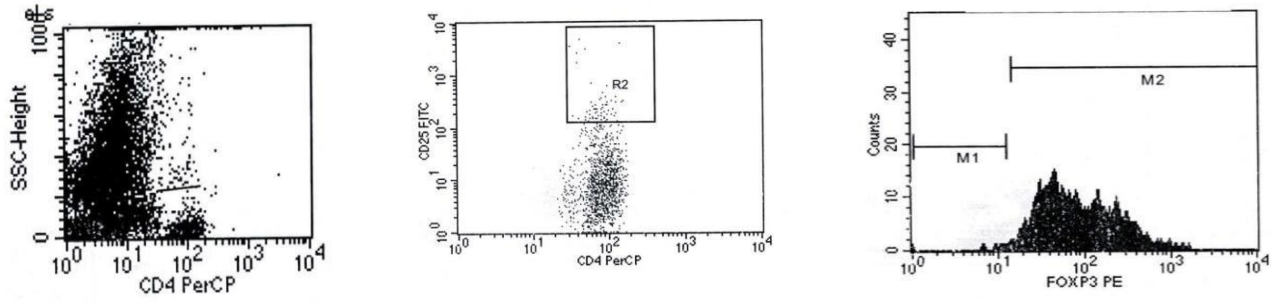
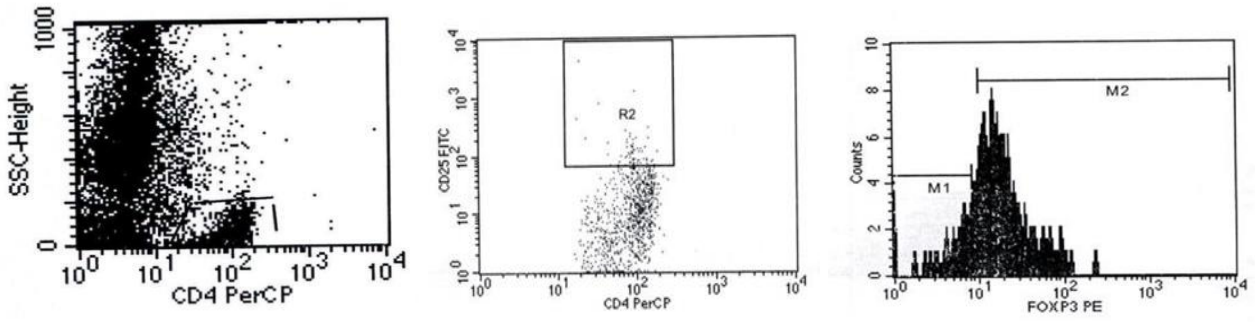


Figure (3 -A & B): flow cytometric analysis showed dot plot of a **good** prognosis case of HCC (A) before treatment FOXP3 (46.67%) &Tregs (3.03%). (B) After treatment FOXP3 (12.82) & Tregs (1.59%).
PBMCS from HCC patients before & after treatment were fixed & stained with anti CD4, CD25 & FOXP3, gating strategies showed population of lymphocytes, which has been selected for detection of CD4 cell to exclude the non-lymphocyte gating on lymphoid cells based on forward side scatter characteristics (A).



R2 is the population of Treg. Histogram plots for FOXP3 on lymphocyte CD4, CD25.
CD4, CD25 population **(B).**

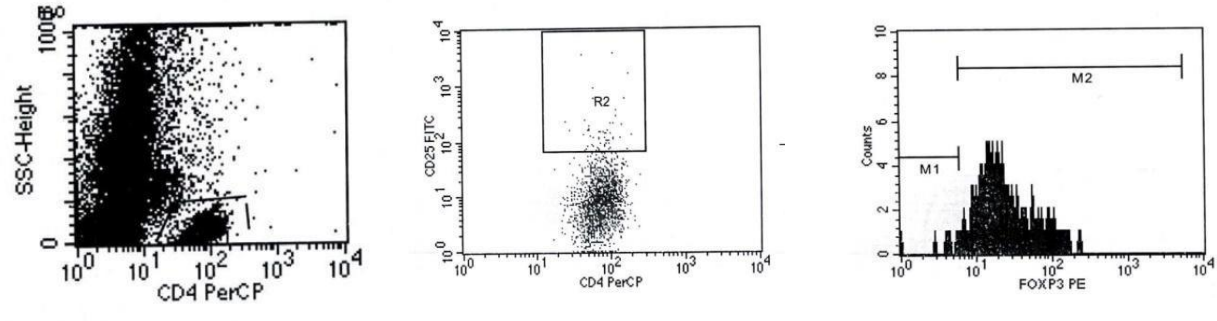


Figure (4- A & B): Flow cytometric analysis showed dot plot of a **bad** prognosis case of HCC (A) before treatment FOXP3 (4.46%) &Tregs (1.91%). (B) After treatment FOXP3 (6.70) & Tregs (1.49%)

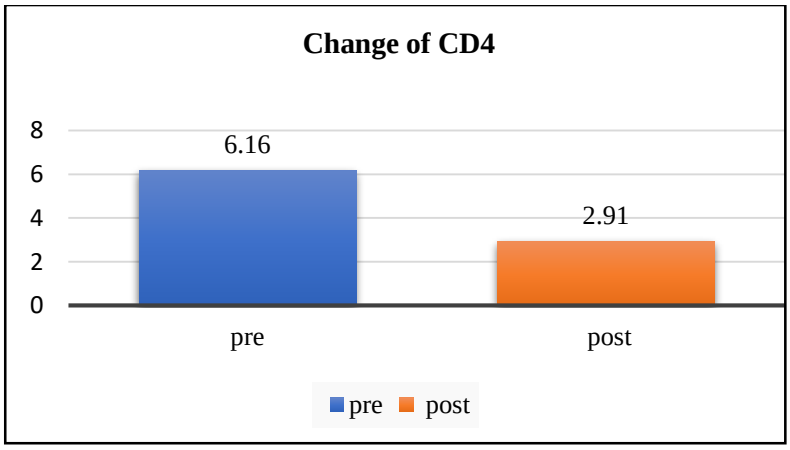


Figure (5): Change assessment of CD4 between pre and post intervention

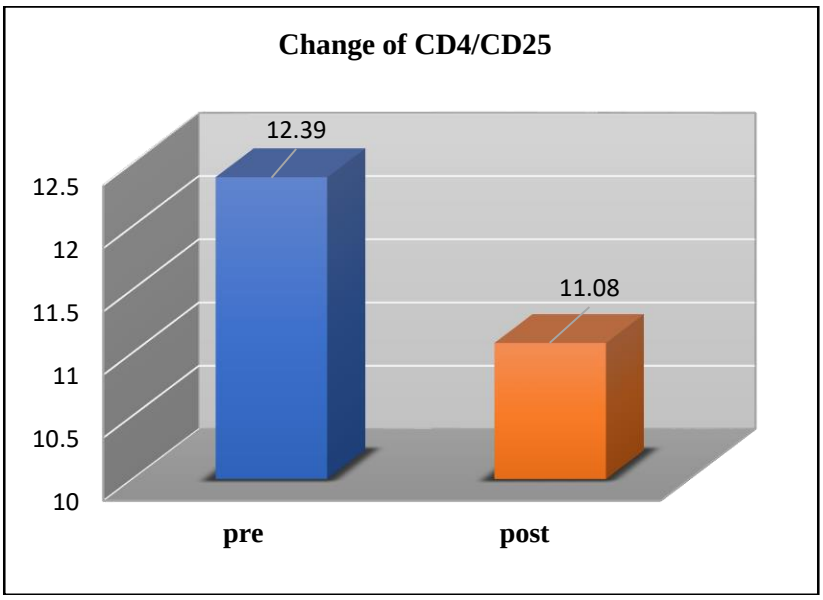


Figure (6): Change assessment of CD4/CD25 between pre and post intervention

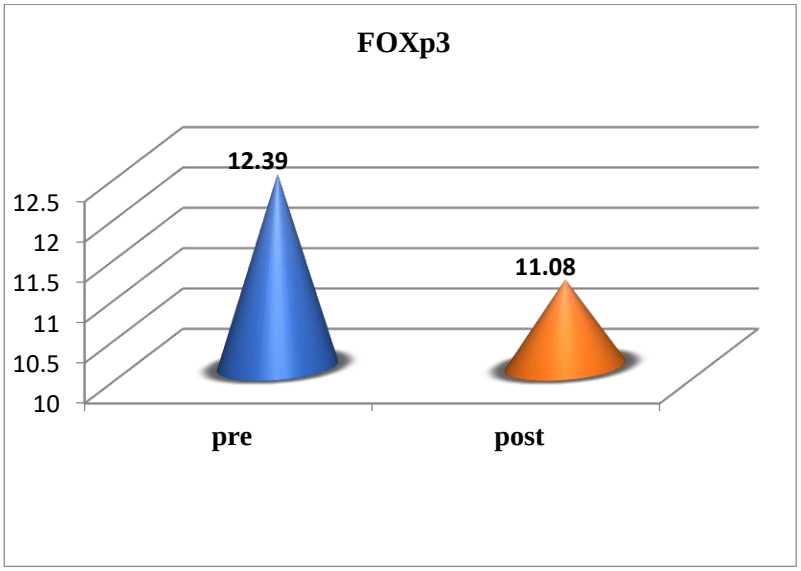


Figure (7): Change assessment of Fox p3 between pre and post intervention

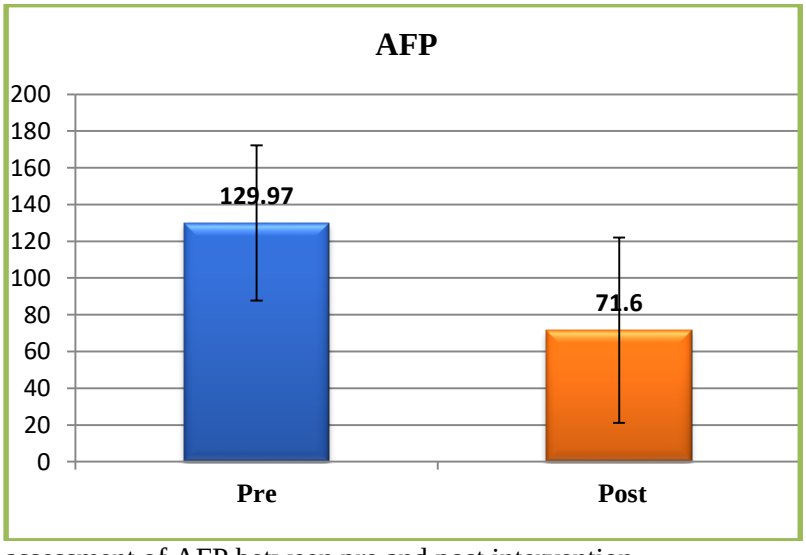


Figure (8): Change assessment of AFP between pre and post intervention

DISCUSSION

Hepatitis C virus (HCV) infection is a major cause of advanced hepatic fibrosis and cirrhosis, with significantly increased risk for development of HCC that is usually present in inflamed fibrotic and/or cirrhotic liver tissue with extensive lymphocyte infiltration. Thus, the nature and localization of tumors-infiltrating lymphocytes (TILs) as CD4+ T cells, CD8+ T cells, and regulatory T cells (Tregs), play a central role in the biologic behavior of HCC [3, 7, 13, 14, 15, 16]. Tregs are an important subset of functionally immune-suppressive T cells, and it has been reported that fork head/winged helix transcription factor P3 (FOXP3) is the most reliable marker of Tregs [17, 10].

The current cohort study showed that age was distributed as 57.44 ± 9.96 with minimum 40 and maximum 76 years old. Regarding sex, males were majority with 72% and female 28%. This can be explained by HCC exhibits sex bias in occurrence higher rate in HBV+ males, and HCV+ females. Also, sex-bias in HCC is partly attributed to sex-specific differences in risk behaviors and environmental exposure. This copes with retrospective study included 300 HCC patients, which was conducted in Egypt by **Elghazaly *et al.*** [18] who revealed that 53% of the studied HCC patients were younger than 60 years old, 81.5% were males and 18.5% were females [19, 20, 21, 22, 23].

As regards prevalence of HCV among studied group, the present study showed that out of 25 patients 56% were HCV positive. **El-Serag** [24] in a cross-sectional and case-control studies revealed that HCV infection (i.e., HCV RNA+ve individuals) has a relative risk for HCC of 15-20 compared to HCV-negative individuals [8, 25, 26].

The present study showed that PLT significantly increased after interventional therapy. This is in agreement with **Chai *et al.*** [27] who reported that PLT significantly was increased after interventional therapy.

Formerly, in the present study, increased Tregs in HCC carcinoma patient's peripheral blood was found. Similar finding is reported by **Tu *et al.*** [19], who study regulatory T cells, especially ICOS+ FOXp3+regulatory T cells, and found that they were increased in the hepatocellular carcinoma microenvironment and predicted reduced survival [7, 10, 28, 29].

Also, the present study showed that CD4 significantly increased but CD4/CD25, FOXp3 significantly decreased in cells that are the major components of TILs. Both of them have unique functions in tumors immunity. Therefore, TILs are always considered as an indicator of host immune reactions against cancers because it has been demonstrated that the infiltrating grade of TILs is correlated with a better prognosis in patients with several types of cancer. For HCC, the prognostic value of CTL infiltration remains controversial.

In this study, the number of CD3+, CD4+ T cells or CD3+, CD8+ T cells did not affect the prognosis of HCC patients. Perhaps, the impaired function of intratumor TILs led to these results. However, our study showed that, patients with high numbers of FOXp3+ cells, and patients with higher FOXp3+ Tregs /CD4+ T cell ratio had worse overall survival rates, with good prognostic value for TIL in HCC. This is going in hand with **Jin *et al.*** [7] who found that their frequency in intratumor tissues had a negative impact on the overall survival of HCC patients. Additionally, higher FOXp3+Tregs/CD4+ T cell and FOXp3+Tregs/CD8+ T cell ratios were also associated with worse prognosis [30, 31, 32]. Nevertheless, **Asmaa *et al.*** [33] revealed that the percentage of peripheral $CD8^{+}CD25^{High+}FOXp3+T$ cells in HCC patients was significantly higher. FOXp3 expression of $CD8^{+}CD25^{High+}$ T cells is higher than that of $CD8^{+}CD25^{low+}$ and $CD8^{+}CD25^{Negative}$ T cells. Study of **Tu *et al.*** [19] potentiates our results where they reported that survival analysis revealed that the FOXP3+Tregs /CD4+ T cell ratio was an independent prognostic factor for HCC. Patients with a higher intratumorally Treg density had a 3.1-fold higher risk of death than those with a lower FOXp3+Tregs/CD4+ T cell ratio. Thus, high-ratio of intratumorally Tregs created a generalized immunosuppressive microenvironment and contributed to the ability for tumor cells to escape the host's immune surveillance [28, 34]. The high number of FOXp3+Treg tumor-infiltration in the liver carcinoma microenvironment raises the question of their recruitment. There are mainly two possible pathways to explain this result; one of the possible ways is by priming naive CD4+ T cells to differentiate into Tregs as reported in gastric and papillary thyroid cancer. The other way is that Tregs selectively migrate to tumors, which is mediated mainly by chemokines and their receptors [28, 35].

CONCLUSION

Our data suggest that Tregs are significantly enriched in HCC and mostly are FOXp3+Tregs. The higher Treg levels indicated a worse prognosis, and the FOXp3+Tregs/CD4+ T cell ratio was an independent prognostic factor for overall survival, promising novel approach for HCC treatment.

Furthermore, more future studies are needed to investigate the role of CD4+, CD25+, FOXp3+Regulatory T cells in patients with HCC and their correlation with the prognosis.

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