Changes in CD4 and CD8 after Interventional Management of Hepatocellular Carcinoma

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Key words:

CD4, CD8, CD4/CD8 ratio, Hepatocellular carcinoma ablation, RFA, PEI, TACE, MWA

Background and study aim : Hepatocellular carcinoma (HCC) has many curative choices which in some circumstances are equal to or even better than surgery. These strategies for treatment of HCC may induce certain local effects which trigger distinct immunological responses that may have a systemic impact on the natural history of the tumour itself. These responses are validated through the measurement of specific immune cells in the systemic circulation. In this study, we tried to observe and analyze changes in the peripheral immune cells that accompany and follow HCC ablation by different procedures of radiological intervention and compare our results with literature. So, this study may be useful with other criteria in the guidelines for the selection of the optimal therapeutic strategy for each patient.

Patients and Methods: This study was conducted on about 50patients diagnosed with HCC who were referred to Tropical Medicine Department at Mansoura University Hospital, Egypt and 20 healthy volunteers as a control. The therapeutic strategy was selected according to the tumor stage and general condition. RFA was performed for12 cases, PEI for 13, MWA for12 and TACE for 13 cases. All Patients were subjected to full history taking, clinical examination, liver function tests, anti HCV antibodies and HBS antigen by 3rd generation ELISA, serum alpha fetoprotein, abdominal ultrasonography, triphasic abdominal computerized tomography and lymphocyte subset assay by flow cytometry 1 day before, and 3 weeks after the treatment.

Results: Regarding the immunological status between control and HCC patients, there was a demonstrable difference in the number of cells in both groups, as the control group had higher levels of CD4+ and CD8+ values. In the RFA group, CD4+ cells and CD4/CD8 ratio remarkably increased after treatment (P <0.001), and the CD8+ cells significantly decreased (P <0.002) with concomitant increase in the CD4+/CD8+ ratio (P<0.001). In the PEI group, CD4+ cells markedly increased after treatment (P<0.001), but there were no significant differences in CD8+ cells and CD4/CD8 ratio. In the MWA group, CD4+ cells markedly increased after treatment (P<0.001), with increase in CD4/CD8 ratio (P<0.007) but there were no significant differences in CD8+ cells. In the TACE group, the CD4+ cells and CD4/CD8 ratio dramatically decreased after treatment (P<0.001), and the CD8+ cells increased significantly (P<0.001).

Conclusion: Our study has proved to find a relationship between immunity and different models of therapy in HCC patients and demonstrated a positive attitude towards increasing immune cells following ablation technique.

INTRODUCTION

Hepatocellular Carcinoma (HCC) is currently the sixth most prevalent cancer worldwide and the third leading cause of cancer-related death **[1]**. It is one of the leading causes of morbidity and mortality in patients with liver cirrhosis. Furthermore, it has a rapidly rising incidence, largely driven by the burden of advanced Hepatitis C Virus (HCV) and non-alcoholic steatohepatitis (NASH) cases **[2]**.

Prognosis for patients with HCC depends on tumor stage at diagnosis, with curative options available only for patients diagnosed at an early stage **[3,4]**. Unfortunately, two thirds of patients with HCC are diagnosed at an advanced stage, when curative options no longer exist and median survival is less than 1 year **[5]**.

Local ablative therapies such as radiofrequency ablation (RFA), percutaneous ethanol injection (PEI) and Microwave coagulation therapy (MWA) offer potential cure for tumors detected at an early stage in well selected patients. For intermediate-stage HCC, transarterial chemoembolization (TACE) is the mainstay of treatment **[6].** Also; RFA combined with TACE is an efficient and safe treatment that provides overall survival rates similar to those achieved with surgical resection **[7].**

Previous studies have shown that immune responses are inevitable in HCC and the lymphocytes phenotype and proportion are being valuable in predicting the response and prognosis in HCC **[8-9]**. Studies have shown a significant increase in frequency of regulatory T cells in peripheral blood, tumor and ascites of HCC patients **[8]**. Although, naturally occurring anti-tumour immune responses could be detected in patients with HCC, this response fails to control tumour growth. This failure could be because of the suppressive effects exerted by the tumour cells on anti-tumour immune responses **[11]**.

Ablative techniques share, irrespective of their mechanism of induction of cell death, the ability to stimulate the immune system **[12]**. Interventional therapeutic procedures produce local and systemic effect capable of inducing cellular infiltration which in turn has the ability to mediate immunological response capable of combating tumour growth and proliferation **[13]**. These valuable data could lead us in the future to create the so-called tumor vaccination.

This study aimed to investigate changes in the peripheral immune cells of HCC patients following treatment with different interventional strategies including RFA, MWA, TACE and PEI. So, this study may be useful with other criteria in guidelines for the selection of optimal therapeutic strategy for each patient.

PATIENTS AND METHODS

This study was a prospective interventional (Randomized Control Trial) study. It was conducted on 50 patients selected from 65 patients diagnosed with HCC who were referred to Tropical Medicine Department at Mansoura University Hospital, Egypt. The study included20 subjects as control. Patients with HCC were 31 males and 19 females and their age ranged from 42 years to 66 years with mean age of (58.42±5.42). According to Child–Turcotte–Pugh classification, 34 patients were classified as class A and 16 as class B.

HCC diagnosis was confirmed by triphasic abdominal computerized tomography scan or dynamic contrast-enhanced MRI. Diagnosis was based on the identification of the typical hallmark of HCC (hypervascular in the arterial phase with washout in the portal venous or delayed phases).

The inclusion criteria were as follows: (i) Patients exhibiting good compliance and providing informed consent, (ii) Patients with primary HCC and naïve to treatment. (iii)Patients with liver cirrhosis of Child-Pugh class A or B .About 15 patients were excluded from the study because they had one or more of the following exclusion criteria: Patients with metastatic tumor, patients with liver cirrhosis of Child- Pugh class C and patients who refused to participate in the study.

This study included 2 main groups: Group A: included 20 healthy control individuals, Group B: included 50 HCC patients. Group B was subdivided into 4 subgroups, subgroup 1(RFA group): Patients in this group were treated with radiofrequency ablation, subgroup2 (MWA group): Patients in this group were treated with microwave ablation, subgroup 3(PEI group): Patients in this group were treated with percutaneous ethanol injection and subgroup4 (TACE group): Patients in this group were treated with transarterial chemoembolization therapy. An informed consent was obtained before patients were enrolled in the study.

All participants in both groups were subjected to full history taking, clinical examination (general and abdominal examination), liver function tests (serum albumin level, serum bilirubin level and international normalized ratio),anti HCV antibodies and HBS antigen by 3rd generation ELISA, serum alpha fetoprotein level(AFP) ,abdominal ultra-sonography, triphasic abdominal computerized tomography and lymphocyte subset assay.

Lymphocyte subset assay:
Peripheral blood samples from patients (after obtaining informed consent and Institutional Review Board approval) were collected 1 day before treatment and three weeks after treatment in EDTA containing tubes. After incubation of blood sample with a mixture of fluorescence labeled anti-CD3, and anti-CD8 monoclonoal antibodies for 15 minutes, lysis of red blood cells was done using 10Test 3 lysis solution (Immunotech, Beckman Coulter, Marseille, France). Analysis

was done using the EPICS XL flow cytometer (Coulter Electronic, Fl, USA). For all flow cytometric analysis each sample was run with an appropriate isotype control (Mouse IgG, Dako-cytoformation, Denmark) to define the negatively stained cells. The following antibodies were used: fluorescein isothiocyanate (FITC)–labeled anti-CD3,R-phycoerythrin-cyanine 5 (RPE-CY5)-labeled anti-CD4, phycoerythrin (PE)–labeled anti-CD8. The antibodies were purchased from immunotech, Beckman Coulter, Marseille, France.

Statistical analysis

Data were analyzed with SPSS version 21. The normality of data was first tested with one-sample Kolmogorov-Smirnov test. Qualitative data were described using number and percent. Association between categorical variables was tested using Chi-square test. Continuous variables were presented as mean ± SD (standard deviation). The two groups were compared with Student t test while paired t- test were used to compare paired data. Analysis Of Variance (ANOVA test) used for comparison of means of more than two groups.

RESULTS

This study was conducted on 50 patients selected from 65 patients diagnosed with HCC. They were 31 males (62%) and 19 females (38%) and their age ranged from 42 years to 66 years with mean age of (58.42±5.42). According to Child–Turcotte–Pugh classification, 34 patients were classified as class A and 16 as class B.

In our study, the correlation between the child score and immunological condition of patients was statistically non-significant.

Most of patients had HCV infection (49), and only one patient had HBV as a cause of liver cirrhosis and HCC subsequently (Table1).

Our patients performed a variety of clinical interventional procedurs including RFA (12), PEI (13), MWA (12) and finally TACE (13). All patients survived to the date of follow up with no major complications or morbidity (Fig. 1).

Regarding the immunological status between control and HCC patients, their was a demonstratable difference in the number of cells in both groups, as the control group had higher levels of CD4+ and CD8+ values (Table 2).

Changes in the lymphocyte subset after treatment in the subgroups of HCC patients are shown in Table 3which demonstrate the tendency of partial variables changes. In the RFA group, CD4+ cells and CD4/CD8 ratio remarkably increased after treatment ($P<0.001$), and the CD8+ cells significantly decreased ($P<0.002$) with concomitant increase in the CD4+/CD8+ ratio ($P<0.001$). In the PEI group, CD4+ cells markedly increased after treatment ($P<0.001$), but there were no significant differences in CD8+ cells and CD4/CD8 ratio. In the MWA group, CD4+ cells markedly increased after treatment ($P <0.001$), with increase in CD4/CD8 ratio ($P<0.007$) but there were no significant differences in CD8+ cells. In the TACE group, the CD4+ cells and CD4/CD8 ratio dramatically decreased after treatment ($P<0.001$), and the CD8+ cells increased significantly ($P<0.001$).

Statistical comparison between all subgroups shows significant decrease in the levels of AFP after treatment in The PEI group, MWA group and TACE group. ($P=0.05$, 0.033 and 0.043 respectively). The levels of AFP after treatment also decreased in RFA group but this decrease was statistically non-significant ($P=0.136$) (Table 4).

Table (1) : Demographic data of HCC patients.

	RFA (n=12)		PEI(n=13)		MWA(n=12)		TACE(n=13)		Total	P value
	No	%	No	%	No	%	No	%	No	
Sex										
Male	7	58.3	5	38.5	9	75.0	10	76.9	31	X^2= 5.215 P= 0.157
Female	5	41.7	8	61.5	3	25.0	3	23.1	19	
Age										
Mean ± SD	57.50±6.01		59.84±5.91		58.33±4.94		57.92±5.13			F=0.436 P=0.728
Min-Max	42-63		45-66		50-66		50-66			
Etiology										
HCV	12		13		12		12		49	
HBV	0		0		0		1		1	
Child classification										
A	10	83.3	6	46.2	6	50.0	12	92.3	34	X^2= .465 P= .024*
B	2	16.7	7	53.8	6	50.0	1	7.7	16	

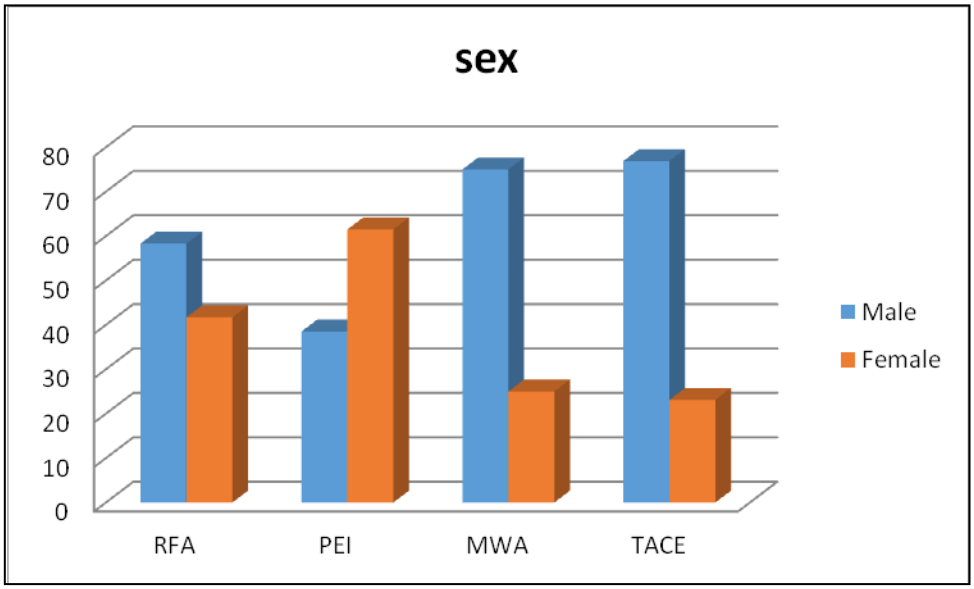

Fig. (1) : Different used methods of ablation

Table (2) : Difference between CD4+ and CD8+ in control and HCC patients

GROUP	CD4+	CD8+	CD4/CD8 ratio
Control	44.61±4.2	31.1±5.51	1.4775±0.07
HCC	35.26±7.15	29.72±6.77	1.2480±0.05
Test of sig. p-value	0.001	0.419	0.02

Table (3) : Immune cells before and after ablation procedures.

Items	Before	After	Test of sig. p-value
	Mean ± SD	Mean ± SD	
RFA group			
CD4	34.41±5.68	43.93±4.42	Paired t-test= 4.902 P=≤.001*
CD8	32.94±6.22	28.27±5.18	Paired t-test= 4.027 P= .002*
CD4/CD8	1.08±0.24	1.61±0.39	Paired t-test= 4.658 P=.001*
PEI group			
CD4	32.5±7.24	40.09±5.66	Paired t-test= 5.316 P=≤.001*
CD8	26.87±7.64	28.67±6.22	Paired t-test= 1.156 P= .270
CD4/CD8	1.27±0.36	1.50±0.59	Paired t-test= 1.715 P= .112
MWA group			
CD4	31.7±3.67	39.89±3.98	Paired t-test= 6.989 P=≤.001
CD8	30.23±6.23	31.81±6.31	Paired t-test= .981 P= .348
CD4/CD8	1.09±0.26	1.30±0.28	Paired t-test= 3.301 P= .007*
TACE group			
CD4	42.11±6.47	35.5±7.70	Paired t-test= 4.699 P= .001*
CD8	29.11±6.15	34.86±6.94	Paired t-test= 4.459 P= .001*
CD4/CD8	1.52±0.45	1.07±0.38	Paired t-test= 4.348 P= .001*

Table (4) : AFP before and after ablation procedures.

	RFA	PEI	MWA	TACE
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
AFP before	43.25±69.73	48.30±53.75	63.22±65.88	75.13±134.95
AFP after	14.859±19.98	25.71±23.20	36.23±32.65	39.08±87.61
Test of sig. p-value	0.136	0.053	0.033	0.043

DISCUSSION

For many years, HCC was regarded as a fatal tumour that has no curative treatment except for surgery, which unfortunately was a rare possibility since we are dealing with a cirrhotic liver with all its surgical limitations and morbid systemic effects, however, in recent years the view has dramatically changed and nowadays we have many curative choices which in some circumstances are equal to and even better than surgery. Hepatic interventional procedures are now considered as a cornerstone in management of HCC due its feasibility, relative safety and affordable cost.

The effect of these procedures is not limited to tumour ablation only, but extends to another immunological local and systemic effect **[14]**. Ablation of tumour cells leads to hepatocyte death, formation of necrotic tissue and establishment of chemical debris, this enhancing

environment confers the release of certain chemicals and cytokines which in turn stimulates immunity and causes sensitization of cellular antigen presentation leading to abortion of further tumour growth and even disappearance of small malignant collections **[15]**.

In this study, we tried to observe and analyze changes in the peripheral immune cells that accompany and follow HCC ablation by different procedures of radiological intervention and compare our results with literature.

Most of HCC lesions were caused by liver cirrhosis due to HCV infection; this is explained by the fact that our community has one of the highest rates of HCV infection worldwide **[16,17]**.

When patients with untreated HCC compared with healthy subjects, they expressed a state of immunological impairment manifested by decreased level of CD4+ and CD4/CD8 ratio with no significant change on CD8+ level. Guan et al, in their study has demonstrated alteration of the immune function following any change of lymphocyte subsets **[13]**. In our study, lymphocytes have significantly changed after ablation according to the method used.

Among the RFA group, 12 patients underwent ablation most of them were Child A (10 patients). All parameters were changed following ablation, there was significant increase in CD4+ and CD4/CD8 ratio ($P<0.001$) which means a strong immunological response due to the presence of necrotic cell death which is much more immunogenic than apoptotic cell death, it leads to inflammatory response that triggers signals that lead to dendritic cell (DC) stimulation and maturation. Our results are equal to Zerbini et al who demonstrated efficient antigen loading following DC maturation which can be elicited locally by heat shock proteins (HSPs), release of cytokines, complements and other inflammatory mediators **[18]**.

Gravante et al, has explained this extensive immunological reaction following RFA by release of "danger signals" which triggers adaptive T-cell responses when an adequate antigen – presenting cell (APC) activation is present. These danger signals consist mainly of HSPs which activates DC [12]. The thermal effect of RFA can skip its local effect to extend to non-specific inflammatory stimulation induced by necrotic cells that might help to overcome immune tolerance or anergy towards the transplanted tumour configuring it as "in vivo tumour vaccination" as mentioned by Hansler et al. **[19]**.

Our work is considered as a unique study for immunological aspect of PEI, since few papers are published in the literature to compare the effect of alcohol ablation therapy on the immunological milieu. 13 patients have received multiple sessions of PEI and have proved to be of significance in elevating CD4+ after ablation ($P<0.001$). Absolute alcohol kills tumor cells by direct cytotoxic effects, causing necrosis of the treated region. It diffuses rapidly into cells, causing cellular dehydration and protein denaturation with resultant coagulation necrosis. This is followed by a fibrotic reaction, thrombosis, and occlusion of small vessels **[20]**. This local effect could be a potent stimulus for the immune cells to attract them to the site of inflammations.

Our results are confirmed by Nakayama et al who has injected ethanol in combination with microwave therapy, interleukins and interferon to treat melanoma and HCC, he has found an increase in the infiltration of T lymphocytes and natural killer (NK) cells into the ablated tissues, confirming that an immune response was elicited **[21]**.

Regarding MWA group, both CD4+ and CD4/ CD8 ratio has significantly increases ($p<0.001$) which are parallel to studies of Dong and his colleagues who had a maximal response on the third day and he also noticed a lower rate of recurrence with high degree of infiltration **[22]**.

Recent studies have proved the effectiveness of TACE in induction of apoptosis, which provides theoretical evidence at the molecular level for the therapeutic effect of TACE [23]**.**In the present study, all the immunologic indicators were significantly changed. When compared with before treatment, in the TACE group, CD4+ cells and the CD4/CD8 ratio markedly decreased and CD8+ cells increased, suggesting that immunologic function was compromised shortly after treatment. These results are extremely similar to other studies especially to the work of Guan and his colleagues who displayed the same results **[13]**.

The discrepancy between the good clinical ablative power of TACE and the impairment of the immunity following the procedure could be explained by the fact that TACE affects blood vessels and lead to its occlusion which essentially could lead to decrease in the flow of the immune

cells, also the toxic materials used in TACE could have a role in this significant change.

Funding: Non.

Conflicts of interest: The authors declare that there is no conflict of interest.

Ethical approval: Informed consent was taken from each patient. The research protocol was duly approved by the Ethical Committee of Faculty of Medicine, Mansoura University **.**

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Editor : Mohamed H Emara; Lecturer of Tropical Medicine, Faculty of Medicine , Zagazig University , Egypt.