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Role of CD8 Cytotoxic T Lymphocytes in Hepatocellular Carcinoma: An Immunohistochemical Study

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Abstract

Background: Hepatocellular Carcinoma (HCC) is the most common primary malignancy of the liver in adults accounting for 85%-90% of liver tumors. It represents the sixth most common malignancy and the fourth leading cause of cancer-related death worldwide. There is an important role of CD8+ tumor infiltrating lymphocytes in host immune defense against tumour progression. Immunotherapy, with modern pharmacologic developments, is a new direction in cancer therapy and therefore immunobiology of hepatocarcinogenesis is under investigation.

Aim of Study: Study expression of CD8 in Hepatocellular carcinoma and its correlation with clinicopathological parameters and explore its effect on HCC prognosis.

Material and Methods: This study included 112 hepatocellular carcinoma cases obtained from the archival material of Pathology Department, National Liver Institute, Menoufia University, between 2010 and 2017. All cases were stained for CD8 antibody. Survival data were available for all HCC cases.

Results: CD8 was expressed in 110 HCC cases (98.2%). There was a statistical significant association between high CD8 percentage and non-cirrhotic adjacent liver (p=0.035). On the other hand, there was a significant association between high CD8 percentage and large tumor size (<5cm) (p=0.015). There was no statistically significant association between CD8 expression and overall survival or recurrence.

Conclusion: We concluded that high CD8 might serve as bad prognostic parameter in HCC as it is associated with non-cirrhotic liver and large tumor size but it doesn't affect HCC overall survival or recurrence.

Key Words: *CD8 – HCC – Immunohistochemistry – Prognosis.*

Introduction

HEPATOCELLULAR Carcinoma (HCC) is the most common primary malignancy of the liver in adults accounting for 85%-90% of liver tumors [1]. It represents the sixth most common malignancy and the fourth leading cause of cancer-related death worldwide [2,3].

In Egypt, liver cancer forms 11.75% of the malignancies of all digestive organs and 1.68% of the total malignancies. HCC constitutes 78.64% of all liver tumors among Egyptians [4]. HCC is the most common cancer in males and the second in females [5,6].

Normally, stroma maintains the tissue homeostasis and acts as a barrier toward tumor formation; however, when a cell starts to be cancerous, its surrounding matrix changes in a way to support cancer development [7,8]. This modified stroma around the malignant cells is termed Tumor Microenvironment (TME) [9].

Tumor Infiltrating Lymphocytes (TILs) are a class of cells that shape the tumor microenvironment and therefore affect carcinogenesis [10]. TILs are considered manifestations of host immune reactions against cancers. Patients with a prominent lymphocyte infiltrate, especially T lymphocytes, who underwent resection for HCC, have reduced recurrence and better survival [11].

There is an important role of CD8+ tumor infiltrating lymphocytes in host immune defense against tumour progression. Studies have indicated a positive correlation between an increased number of CD8+ TILs and the occurrence of tumour cell apoptosis with inhibition of tumor progression [12,13].

Immunotherapy, with modern pharmacologic developments, is a new direction in cancer therapy and therefore the immunobiology of hepatocarcinogenesis is under investigation [10,14].

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The aim of this study is to evaluate the expression of CD8 in HCC cases and its correlation with clinicopathological parameters and explore its effect on HCC prognosis.

Material and Methods

This retrospective case study included 112 Hepatocellular Carcinoma (HCC) cases. All specimens were obtained from Egyptian patients either by partial hepatectomy or total hepatectomy procedures and retrieved from the archival material of Pathology Department, National Liver Institute, Menoufia University, during the period between 2010 and 2017. They were selected according to the availability of the blocks for serial cutting and examination. Survival data were available for all of the studied HCC cases.

Clinical data:

Data were collected from patients' medical records including:

- *Age:* Studied cases were divided into two groups; <60 years and ≥60 years [15].
- Gender.
- *Laboratory investigations:*
  - Serological detection of viral etiology by ELISA or PCR.
  - Serum Alpha Fetoprotein (AFP) (available for 91 cases only) was recorded as the highest value within the 6 months prior to surgical resection [16]. Patients were divided into two groups; <200ng/ml and ≥200ng/ml [17].
  - Child pugh classification (available for 88 cases only) was divided into three classes (A, B and C) according to five clinical measures of liver disease (total bilirubin, serum albumin, prothrombin time or INR, ascitis and hepatic encephalopathy) [18-20].
- *Radiological investigations:* To detect [21]:
  - Tumor site: Unilobar or Bilobar.
- *Type of treatment:* Interferon, Direct Acting Antiviral (DAA) or not received any treatment [22].
- *CLIP prognostic score:* Available for 88 cases only. It includes the number of nodes, Alpha-fetoprotein (AFP) level, portal vein thrombosis and Child-Pugh classification. It was classified from 0-5 [23,24].

Histopathological evaluation: From each representative paraffin block of the studied cases, 4 µm thick sections were cut, stained by haematoxylin and eosin (H & E) and re-evaluated to confirm the diagnosis and to assess the following:

- *Tumor size:* The greatest dimension in centimetres. Cases were divided into two groups; tumor size <5cm and ≥5cm [25].
- *Tumor focality:* Single or multiple [15].
- *Clear cell changes:* It was assessed subjectively as present or absent.
- *Tumor grade (G):* Classic HCC was graded based on the highest grade of tumor differentiation [26]. Grade of classic HCC was then lumped into two groups; low grade (G 1-2) and high grade (G 3-4) [27,28].
- *Pathological stage:* According to American Joint Committee on Cancer (AJCC) staging system, 8th edition [29]. HCC cases were divided into two groups; early stage (T 1-2) and advanced stage (T 3-4) [30].
- *Lymphovascular Invasion (LVI):* LVI was defined as presence of tumor cells within or adherent to the wall of an unequivocal endothelial-lined vascular space (vascular or lymphatic) [31].
- *Adjacent non-tumor liver tissue:* Cirrhotic or non cirrhotic [15].

Tissue Microarray Constructing Technique (TMA):

Three tissue cores with a diameter of 1.5 micron from the selected area in the donor block were punched using a manual tissue arrayer's needle (Breecher Instrument, USA) then arrayed on a recipient block. Tissue Microarray Constructing Technique (TMA) map was created indicating the position and origin of each core in the tissue microarray. A control normal tissue was placed in strategic regions throughout the blocks [32]. Five micron thick sections were cut from recipient block and placed on positive charged slides and used for immunohistochemical staining.

Immunohistochemistry:

Paraffin-embedded tissue sections (5mm) were deparaffinized in xylene and rehydrated. The sections were treated with 200ml of tris-EDTA high PH retrieval solution (Dako, Ref K8000, Glostrup, Denmark) for 20 minutes. Endogenous peroxidase was blocked with peroxidase-blocking reagent. The primary antibody was CD8 [Clone C8/144B, A monoclonal mouse anti-human antibody (Dako Denmark. A/S)] which was ready to use.

A positive reaction was revealed using substrate-chromogen solution (DAB). The sections were

then counterstained with Mayer's hematoxylin. Positive controls for the reaction were performed with paraffin-embedded sections of normal human tonsil and negative controls were used for each run of immunohistochemical staining by omitting the primary antibody.

Interpretation of immunostaining results:

Assessments of immunohistochemical results were determined using a semiquantitative visual approach. Unintentional bias was prevented by coding patients' slides.

Positive cells were identified by the presence of brownish membranous coloration detected by DAB reaction. The staining intensity was also reported and scored from 0 to 3 (0=Negative, 1= Mild staining, 2=Moderate staining and 3=Strong staining) for each cells. The staining intensity score 2 and 3 were classified as positive. 20% was used as cutoff point for percentage of positive lymphocytes and cases were divided into two groups; low percentage (0-20%) and high percentage (21-100%) **[15]**.

Overall survival and recurrence data:

Overall survival time and recurrence were available for all 112 patients.

- *Overall survival:* OS was calculated from the date of surgery to either the date of death or the last follow-up **[33]**.
- *Recurrence:* The diagnosis of recurrence was based on typical imaging appearance with or without an elevated serum AFP after the date of surgery **[34,35]**.

Statistical analysis:

Data were fed to the computer and analyzed using Statistical Package for the Social Sciences (SPSS) software package Version 20.0. (Armonk, NY: IBM Corp). Qualitative data were described using number and percent.

Chi-Square (χ^2) test and Fisher Exact (FE) test were used for categorical variables, to compare between different groups. Mann-Whitney (U) test and Kruskal-Wallis (H) test were used for abnormally distributed quantitative variables, to compare between two or more studied groups. Kaplan Meier test was constructed to differentiate survival and recurrence between compared groups using the log rank test. $p \leq 0.05$ was considered to indicate statistical significance in all tests **[36]**.

Results

Table (1): Clinicopathological characteristics of the studied HCC cases.

Variables		HCC (No.=112)	
		No	%
Age:	<60	75	67.0
	≥60	37	33.0
	Min.-max.	35.0-75.0	
	Mean ± SD.	56.50±7.09	
	Median	57.0	
Gender:	Male	91	81.2
	Female	21	18.8
	M:F	4.3:1	
Etiology:	Viral	101	90.2
	Non viral	11	9.8
AFP (ng/ml) (No=91):	Min.-max.	1.0-20010.0	
	Mean ± SD.	930.5±3070.5	
	Median	33.0	
AFP (ng/ml) (No=91):	<200 (ng/ml)	71	78.0
	≥200 (ng/ml)	20	22.0
Child pugh classification (No= 88):	A	58	65.9
	B	30	34.1
	C	0	0
Focality:	Single	80	71.4
	Multiple	32	28.6
Site:	Unilobar	106	94.6
	Bilobar	6	5.4
Treatment:	Interferon	24	21.4
	DAA	49	43.8
	No	39	34.8
CLIP prognostic score system (No=88):	0	20	22.7
	1	42	47.7
	2	10	11.4
	3	11	12.5
	4	4	4.5
	5	1	1.2
Size:	Min.-max.	1.0-17.0	
	Mean ± SD.	5.19±3.11	
	Median	4.0	
	<5cm	69	61.6
	≥5cm	43	38.4
Clear changes:	Yes	9	8.0
	No	103	92.0
Grade:	I	1	0.9
	II	76	67.9
	III	31	27.6
	IV	4	3.6
Combined grade:	I/II	77	68.8
	III/IV	35	31.2
Pathological stage:	T1	44	39.3
	T2	55	49.1
	T3	12	10.7
	T4	1	0.9
Combined pathologic stage:	Early (T1/T2)	99	88.4
	Advanced (T3/T4)	13	11.6
Lymphovascular invasion:	Present	49	43.8
	Absent	63	56.2
Adjacent liver:	Cirrhotic	92	82.1
	Non cirrhotic	20	17.9

HCC : Hepatocellular Carcinoma.
No : Number.
% : Percentage.
SD : Standard Deviation.
Min. : Minimum.
Max. : Maximum.
M/F : Male to female ratio.
AFP : Alpha fetoprotein.
DAA : Direct Acting Antiviral.
CLIP score : Cancer of the liver Italian program score.

Clinicopathological characteristics of the patients: Clinicopathological characteristics of the selected cases are showed in (Table 1).

CD8 immunohistochemical results:

CD8 was expressed in tumor infiltrating lymphocytes of 110 HCC cases (98.2%) Fig. (1A), (Table 2).

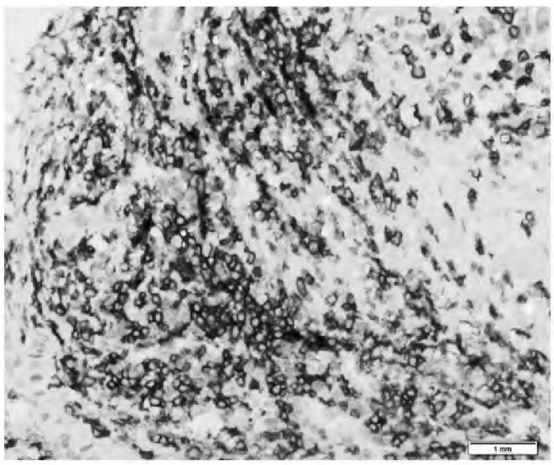
Table (2): Immunohistochemical expression of CD8 in the studied HCC cases.

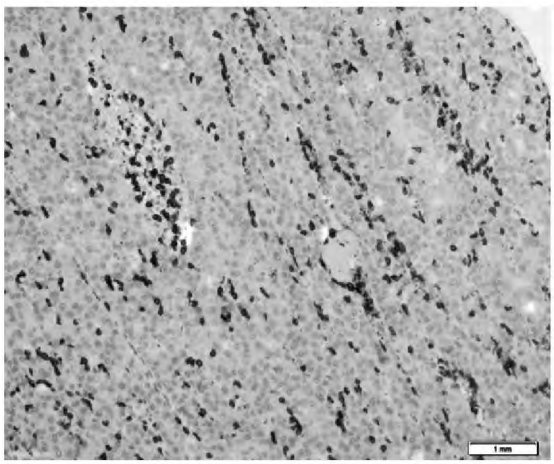
Variables	HCC (No.=112)	
	No	%
CD8 expression:		
Negative	2	1.8
Positive	110	98.2
CD8 intensity:		
0	2	1.7
1	0	0.0
2	21	18.8
3	89	79.5
Counting score:		
0	23	20.5
1	35	31.3
2	54	48.2
CD8 percentage:		
Low percentage (0-20)	63	56.2
High percentage (21-100)	49	43.8
Min.-max.	0.0-90.0	
Mean ± SD.	26.96±22.33	
Median	20.0	

HCC : Hepatocellular Carcinoma.
No : Number.
% : Percentage.
SD : Standard Deviation.
Min. : Minimum.
Max. : Maximum.

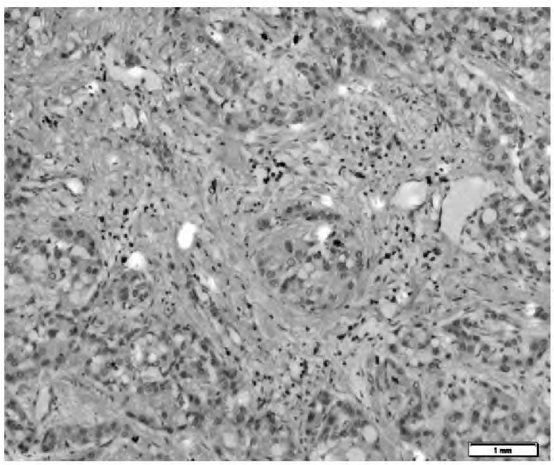
A statistically significant association was found between high CD8 percentage and bad prognostic parameters as adjacent non cirrhotic liver (p=0.035) (Table 3), Figs. (1B,C). Also, high CD8 percentage was statistically associated with large sized tumors (<5cm) (p=0.015) (Table 3).

Using univariate survival analysis, there was no statistically significant association between CD8 expression and overall survival or recurrence (Table 4).





(B)



(C)

Fig. (1): (A) A case of HCC with positive membranous expression of CD8 T lymphocytes (IHC X400). (B): A case of HCC with adjacent non cirrhotic liver showing high CD8 percentage of expression (IHC X200). (C): A case of HCC with adjacent cirrhotic liver showing low CD8 percentage of expression (IHC X200).

Table (3): Relationship between CD8 percentage of expression and clinicopathological parameters in the studied HCC cases.

Variables	CD8 percentage				χ^2	*p*
	Low (0-20) (n=63)		High (21-100) (n=49)			
	No	%	No	%		
Age:						
<60	46	61.3	29	38.7	2.3 84	0.123
≥60	17	45.9	20	54.1		
Gender:						
Male	53	58.2	38	41.8	0.782	0.376
Female	10	47.6	11	63.6		
Etiology:						
Viral	59	58.4	42	42.3	1.960	0.161
Non viarl	4	36.4	7	36.4		
AFP (ng/ml):	(n=52)		(n=39)			
<200 (ng/ml)	41	57.7	30	57.7	0.048	0.826
≥200 (ng/ml)	11	55.0	9	45.0		
Child pugh classification:	(n=51)		(n=37)			
A	36	62.1	22	37.9	1.182	0.277
B	15	50.0	15	50.0		
C	0	0	0	0		
Focality:						
Single	42	52.5	38	47.5	1.600	0.206
Multiple	21	65.6	11	34.4		
Site:						
Unilobar	59	55.7	47	44.3	0.280	*p*=
Bilobar	4	66.7	2	33.3		0.697
Alive/dead:						
Alive	33	55.0	27	45.0	0.082	0.775
Dead	30	57.7	22	42.3		
Treatment:						
Interferon	11	45.8	13	54.2	4.409	0.110
DAA	33	67.3	16	32.7		
No	19	48.7	20	51.3		
CLIP prognostic score system:	(n=51)		(n=37)			
0	10	50.0	10	50.0	3.658	*p*=
1	24	57.1	18	42.9		0.660
2	7	70.0	3	30.0		
3	8	72.7	3	27.3		
4	2	50.0	2	50.0		
5	0	0.0	1	100.0		
Size:						
<5cm	45	65.2	24	34.8	5.873*	0.015*
≥5cm	18	41.9	25	58.1		
Clear changes:						
Yes	4	44.4	5	55.6	0.554	*p*=
No	59	57.3	44	42.7		0.501
Grade:						
I	0	0.0	1	100.0	1.544	*p*=
II	44	57.9	32	42.1		0.762
III	17	54.8	14	45.2		
IV	2	50.0	2	50.0		
Combined grade:						
I/II	44	57.1	33	42.9	0.080	0.778
III/IV	19	54.3	16	45.7		
Pathological stage:						
T1	21	47.7	23	52.3	3.456	*p*=
T2	35	63.6	20	36.4		0.292
T3	6	50.0	6	50.0		
T4	1	100.0	0	0.0		
Combined pathologic stage:						
Early (T1/T2)	56	56.6	43	43.4	0.035	0.853
Advanced (T3/T4)	7	53.8	6	46.2		
Lymphovascular invasion:						
Present	31	63.3	18	36.7	1.742	0.187
Absent	32	50.8	31	49.2		
Adjacent liver:						
Non cirrhotic	7	35.0	13	65.0	4.468*	0.035*
Cirrhotic	56	60.9	36	39.1		

HCC : Hepatocellular Carcinoma.
No : Number.
% : Percentage.
AFP : Alpha Fetoprotein.
DAA: Direct Acting Antiviral.
CLIP score: Cancer of the liver Italian program score.
χ^2 : Chi square test.
p : *p*-value for comparing between the studied categories.
* : Statistically significant at $p \leq 0.05$.

Table (4): Univariate overall survival and recurrence of HCC cases according to CD8 expression.

Variable	Overall survival (months)			
	Mean survival time	SE	Log rank	*p*-value
CD8:				
Negative	24.0	24.0	0.053	0.817
Positive	34.53	30.35		
Variable	Recurrence (months)			
	Mean recurrence time	SE	Log rank	*p*-value
CD8:				
Negative	18.0	0.0	0.632	0.427
Positive	9.727	0.990		

Discussion

Our study showed no correlation between CD8 expression and clinicopathological parameters rather than adjacent non cirrhotic liver tissue and large tumor size. Giuşcă et al., (2015) indicated the difficulty to confirm the direct connection of lymphocytes to the clinical behavior of the tumor [37].

In current study, there was a statistically significant association between high CD8 percentage and adjacent non cirrhotic liver (p=0.035). This agreed with Freeman et al., (2003) who reported that there was no relationship between hepatic lobular infiltrated cytotoxic T lymphocytes activity and histological evidence of liver damage. These findings indicated that CD8 T lymphocytes may not play a role in occurrence of severe fibrosis of the liver [38]. Furthermore, Desai et al., (2019) demonstrated that HCC that developed in non-cirrhotic liver presents at an advanced stage as HCC in non-cirrhotic patients is clinically silent in its early stages because of lack of symptoms and surveillance imaging [39]. Also, approximately 25% of non-cirrhotic HCC present with extra-hepatic metastasis [40]. The recurrence rate of HCC in non-cirrhotic liver is extremely high [41]. In context of these results, high CD8 expression is associated with bad prognostic parameter such as non-cirrhotic adjacent liver. This may be explained by the immune scape strategies of tumor cells such as immunoediting, downregulation of MHC molecule expression and presence of inhibitory molecules [42].

In our study, there was a significant association between high CD8 percentage and large tumor size (<5cm) (p=0.015). This can be explained by depletion of CD8 T lymphocytes during process of inhibition of tumor progression. This opposes AN et al., (2014) who reported that the average numbers of CD8 T cells in the tumor parenchyma and stroma were higher in patients with tumor diameters ≤ 5cm than in patients with tumor diameters >5cm, so CD8 is involved in HCC diameter control [43].

On the other hand, our study showed no correlation between CD8 expression and overall survival or recurrence of the tumor. This agreed with Giuşcă et al., (2015) who showed that the absence of the correlation between TILs and survival in HCC can be due to intense loss of lymphocytes through the proapoptotic processes that develop in the microenvironment of liver parenchyma [37]. Chang et al., (2017) also found that the predictive role of CD8+ TIL for recurrence or survival was not demonstrated [15]. On the other hand, Gabrielson et al., (2016) revealed that a high density of CD8+ cells in the tumor experienced a significant reduction in the rate of HCC recurrence [44] and this difference might be explained by different antibody clones used. However, accumulating evidence has indicated that the activation state of CTLs, instead of just the existence of CTLs, were of great prognostic significance [45-47].

Conclusion:

This study showed that there is a significant association between high CD8 cytotoxic T lymphocytes expression and bad prognostic parameters such as non-cirrhotic adjacent liver to the tumor and large tumor size, but there is no correlation between CD8 cytotoxic T lymphocytes and overall survival or recurrence.

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دور الخلايا التائية (سى دى ٨) فى سرطان الخلايا الكبدية، دراسة هيستوكيميائية مناعية

\المقدمة: سرطان الكبد هو أكثر السرطانات الخبيثة الكبدية الأولية شيوعاً بين البالغين بنسبة تتراوح بين ٨٥٪ إلى ٩٠٪ وهو سادس أكثر أنواع السرطانات إنتشاراً، والسبب الرابع الأكثر شيوعاً للوفاة بسبب السرطان. هناك دور هام للخلايا الليمفاوية المصاحبة للورم (سى دى ٨) فى الدفاع ضد تطور الورم. يعتبر العلاج المناعى، مع التطورات الدوائية الحديثة، هو نوع جديد فى علاج السرطان، وبالتالى فإن البيولوجيا المناعية لتسرطن الخلايا الكبدية قيد التحقيق.

\الأهداف: دراسة تعبير الخلايا التائية (سى دى ٨) فى سرطان الخلايا الكبدية وعلاقتها بالمؤشرات السريرية والمرضية وإكتشاف تأثيرها على تطور سرطان الخلايا الكبدية.

\الطرق: هذه الدراسة إشتملت على ١١٢ حالة سرطان خلايا كبدية تم الحصول عليهم من المادة الأرشيفية بقسم الباثولوجى، معهد الكبد القومى، جامعة المنوفية فى الفترة ما بين ٢٠١٠ و٢٠١٧. جميع الحالات تم صباغتها بواسطة سى دى ٨. جميع بيانات معدل الوفاة كانت متاحة لجميع حالات سرطان الخلايا الكبدية.

\النتائج: سى دى ٨ تم التعبير عنها فى ١١٠ حالة سرطان خلايا كبدية بنسبة ٩٨.٢٪. تم تأكيد إرتباط نسبة التعبير المرتفعة لسى دى ٨ بنسيج الكبد الغير متليف المجاور للورم (p=0.35). على الجانب الآخر تم تأكيد إرتباط نسبة التعبير المرتفعة لسى دى ٨ بحجم الورم الكبير (>٥سم) (p=0.015). كما أنه لم يتم تأكيد إرتباط تعبير سى دى ٨ بمعدل الوفاة أو بتجدد حدوث الورم.

\الخلاصة: خلصنا إلى أن سى دى ٨ ربما تعمل كمؤشر سئ لسرطان الخلايا الكبدية لأنها ترتبط بالكبد الغير متليف المجاور للورم وحجم الورم الكبير لكنها لا تؤثر على معدل الوفاة أو تجدد حدوث الورم.