FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 53, No. 3, 2015
pp. 272–281

ORIGINAL PAPER

VIA MEDICA

Comparative analysis of CD4 and CD8 lymphocytes — evidences for different distribution in primary and secondary liver tumors

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Abstract

Introduction. The impact of tumor cells on tumor-infiltrating lymphocytes (TILs) in cancer development is not yet clarified. Our study analyzed the distribution and prognostic value of CD4+ and CD8+ T lymphocytes in hepatocellular carcinoma (HCC) and liver metastases (LM).

Material and methods. Archival tissue specimens of 35 HCC and 39 LM patients were immunohistochemically processed. The number of intratumoral (IT) and peritumoral (PT) CD4+ and CD8+ T cells was quantitatively analyzed.

Results. We noted large variances of T lymphocyte subpopulations. Similar number of CD4+ and CD8+ lymphocytes was present in HCC, whereas in LM the number of CD8+ cells was approximately two times higher than CD4+ lymphocytes. A significant prevalence of T cells in PT over IT areas was observed. The prognostic value was demonstrated only for PT CD8+ lymphocytes in LM, their reduced number being associated with shorter survival.

Conclusions. The differences between proportions of T lymphocytes within tumor and its environment might be explained by proapoptotic effect of cancer cells on TILs. *(Folia Histochemica et Cytobiologica 2015, Vol. 53, No. 3, 272–281)*

Key words: hepatocellular carcinoma; liver metastases; Th cells; Tc cells; prognostic factors

Introduction

Characterization of tumor microenvironment holds a key role in the attempt to understand the mechanism of primary and secondary liver carcinogenesis, its immune component, responsible for the development of an immune response of the host against the tumor, being the main point of interest [1–4]. The largely inconsistent biological behavior of primary and secondary liver tumors could be also explained by the settled relationship between the tumoral component and microenvironment [1, 5, 6].

Although the role of lymphocytes in immunity was stated for the first time over one hundred years ago, the first report which proved the correlation between the increased number of cytotoxic CD8+ T lymphocytes and the favorable prognosis in melanoma tumor pathology had been published in 1989 [7]. The study on colorectal cancer showed the involvement of T lymphocytes in destruction of cancer cells within tumor and called them tumor-infiltrating lymphocytes (TILs) [8].

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The ratios between CD4+ and CD8+ T lymphocytes within TILs are variable, depending not only on the investigated tumor type, but also on the quantification method. However, numerous studies on colorectal [9], ovarian [10], breast [11, 12], and esophageal cancer [13], leukemia [14], and other malignancies confirmed the protective prognosis value of the immune infiltrate with CD8+ T lymphocytes [15]. Nevertheless, there are conflicting results, such as in melanoma, where TILs influence survival either positively [16, 17] or negatively [18, 19].

All these data support the fact that despite concerted efforts towards the decoding of a stable pattern in the relationship between the presence of TILs and tumor behavior, the dilemma regarding the position of TILs as friend or foes [20] is still present. The review of the literature reveals attentiveness to the investigation of TILs in hepatic parenchyma, in primary tumor context — namely hepatocellular carcinoma (HCC) [3, 21–32] or in liver metastases (LM) [33–35]. Nevertheless, we have to highlight the fact that none of the published studies presented comparative data of CD4+ and CD8+ T lymphocytes in primary *versus* secondary liver tumors.

Within this framework, our study focused on the investigation of the immune infiltrates in HCC and LM, using a comparative evaluation of intratumoral and peritumoral CD4+ and CD8+ T lymphocytes, respectively, with the aim to ascertain the value of their ratio as a prognostic factor.

Material and methods

Patients. The studied material consisted of paraffin-embedded tissue specimens corresponding to 35 patients with HCC (Group 1) and 39 patients with LM (Group 2) diagnosed and surgically treated between January 2009 and December 2011 at "Sf. Spiridon" University Hospital, Iaşi.
The study was approved by the Ethics Committee of the University of Medicine and Pharmacy "Grigore T. Popa", Iaşi, Romania.

The clinicopathological features are presented in Table 1; the tumor stage and histological grade both for HCC and LM were established according to the criteria of pTNM classification [36]. The patients did not receive preoperative chemotherapy or radiotherapy.

All documented patients' deaths were related to previously diagnosed cancer disease. The median survival rates were 5 months for HCC and 17 months for LM cases. According to follow-up records, 36.12% of HCC patients and 27.78% of LM patients were alive at the 31st of January 2013.

Immunohistochemistry. For each case, a single paraffin-embedded block, considered as representative for the tumoral features and inflammatory infiltrate was chosen. Immunohistochemical (IHC) staining was performed on 4-μm-thick paraffin sections placed on coated microscopic slides, specially treated for immunohistochemistry (Thermo Fischer Scientific, Fitchburg, WI, USA). The slides were then dried at room temperature (RT) and heated in an oven at (58°C for 60 min, for a greater adherence of the tissue. Standard protocol for IHC technique was used. First, the slides were dewaxed in two baths of xylene and hydrated in four baths of graded alcohol (100%, 90%, 80%, 70%), 10 min each, than rinsed in distilled water. To unmask the antigenic site, slides were placed in antigen retrieval solution pH 6.0 (citrate-based buffer — Novocastra Epitope Retrieval Solutions Leica Biosystem, Newcastle Ltd, United Kingdom) and heated at 98°C for 30 min (HIER technique). After blocking the endogenous peroxidases in 3% hydrogen peroxide solution, for 10 min, slides were incubated with the primary antibodies, overnight at 4°C, in a humidified chamber. We used anti-CD4 (Clone 4B12, code NCL-L-CD4-368, Novocastra; dilution 1:40) and anti-CD8 (Clone 1A5, code NCL-L-CD8-295, Novocastra; dilution 1:80). For the horseradish peroxidase (HRP) enzymatic detection of reaction the slides were incubated with the secondary antibody and the polymer for 30 min each, at RT (Novolink polymer kit, Novocastra). Reaction was developed with DAB (3.3'-diaminobenzidine dihydrochloride) chromogen, for 5 min at RT, till a brown stain appeared. Slides were then rinse in running tap water for 5 min and counterstained with hematoxylin. Negative (omitting the incubation with the primary antibody) and positive (tonsil) controls were run in the same staining session.

Quantitative analysis. TILs were assessed by using an adapted methodology [37]. The number of CD4+ and CD8+ T lymphocytes, respectively, was counted in 10 microscopic fields per each section (slide) corresponding to each case at total magnification of × 200, independently by two histopathologists (S.E.G. and E.R.A.) for intratumoral (IT) and peritumoral (PT) areas. The peritumoral areas were defined outside the proper tumor field areas, at the border between the tumoral cells and hepatocytes' layers belonging to the normal liver parenchyma. A mean value/case was calculated for each lymphocytes subtype and territory. Subsequently, by using the mean values/case, we computed the mean value for Group 1 and Group 2, for CD4+ and CD8+ T lymphocytes, and IT and PT territory, respectively.

Statistical analysis. Mean values of either CD4+ or CD8+ cells in respective territories were used as threshold in order to obtain subgroups including cases with lower and, respectively, higher CD4+/CD8+ lymphocytes mean value/case than the threshold. Statistical analysis has been performed using MedCalc software (MedCalc Software, Ostend, Belgium) and GraphPad Prism — ver. 6.05 (GraphPad Software Inc., La Jolla, CA, USA). Patients' characteristics were

Table 1. Clinicopathological characteristics of patients with hepatocellular carcinoma and liver metastases

Clinicopathological characteristics	Variable description	Patients' number	%
Hepatocellular carcinoma			
Age (years)		62.9 ± 10.7; 64; 23–83*	
≤ 63		17	48.57
> 63		18	51.43
Gender			
Female		12	34.29
Male		23	65.71
Tumor stage**			
Stage I	T1N0M0	5	14.29
Stage II	T2N0M0	13	37.14
Stage IIIa	T3N0M0	16	45.71
Stage IV	TxNxM1	1	2.86
Histological grade**			
G1	Well differentiated	13	37.14
G2	Moderately differentiated	15	42.86
G3	Poorly differentiated	7	20.00
Liver metastases			
Age (years)		67.6 ± 11.9; 70; 35–86*	
≤ 68		17	43.59
> 68		22	56.41
Gender			
Female		18	46.15
Male		21	53.85
Tumor stage			
Stage IV	TxNxM1	39	100
Histological grade			
G1	Well differentiated	3	7.69
G2	Moderately differentiated	20	51.28
G3	Poorly differentiated	14	35.90
G4	Undifferentiated	2	5.13
Tumoral extension			
One lobe		26	66.67
Many lobes		13	33.33
Origin			
	Colon	18	46.15
	Rectum	6	15.39
	Duodenum	1	2.56
	Stomach	8	20.53
	Pancreas	2	5.13
	Gallbladder	1	2.56
	Adrenal gland	1	2.56
	Breast	1	2.56
	Ovary	1	2.56

*Mean ± SD; median; range; **pTNM stages (IIIb, IIIc) and G4 were not diagnosed, and, therefore, not shown

given in absolute and relative numbers or as mean values with standard error of the mean (SEM). Mean lymphocytes values were further checked with the use of D'Agostino normality test, and the Fisher 2 × 2 exact test was used to analyze relationships between the subgroups. Mann-Whitney U test was applied to check the statistical differences between two groups. Spearman's correlation was utilized for testing the associations between variables. The Cox-Mantel proportional hazard regression model was used to evaluate the effect of explorative variables on survival of HCC and LM patients. Firstly, univariate Cox regression analysis for every single variable was performed. Secondly, variables with a p value < 0.05 were included into multivariate Cox regression analysis with a variable selection *via* backward

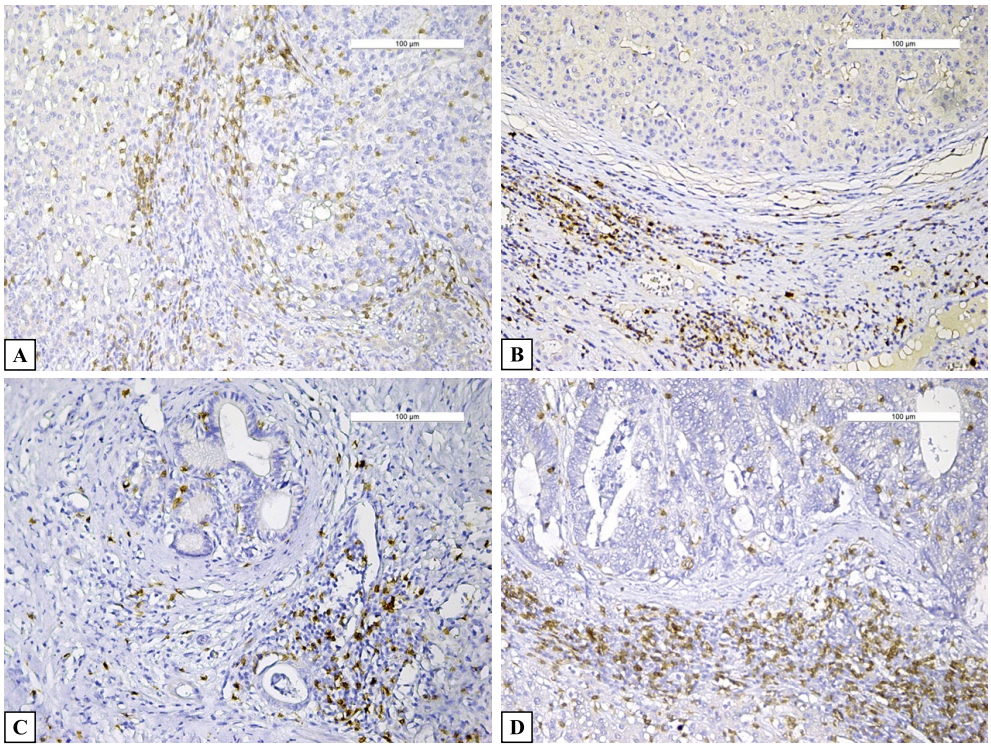

Figure 1. The distribution of T lymphocytes in hepatic tumors' environment shows similar pattern in hepatocellular carcinoma (**A:** CD4+ cells, **B:** CD8+ cells) and liver metastases (**C:** CD4+ cells, **D:** CD8+ cells). Total magnification × 200

elimination. All associations were presented as hazard ratios (HR) with their 95% confidence interval (CI) and *p* values. Variables for overall survival (OS) and progression-free survival (PFS) rates were calculated separately. Kaplan-Meier estimations were performed to describe survival rates.

Results

Qualitative analysis of T cells populations in HCC and LM

The microscopic examination revealed both in HCC (Group 1) and LM (Group 2) a similar distribution pattern for CD4+ and CD8+ T lymphocytes, with an obvious cellular predominance in the PT area, especially at the border with normal liver parenchyma (tumor's invasion front), compared with IT localization (Figure 1A–D). We noted a specific PT arrangement of CD4+ and CD8+ T lymphocytes, around the tumoral islands, as condensed layers or mantle zones, or as compact structures resembling lymphoid nodules, whereas they were isolated or dispersed within the tumoral mass.

Quantitative analysis of T cell populations in HCC and LM

We found similar number of CD4+ and CD8+ cells in HCC (CD8+ mean value 148.5 ± 114.03 *vs.* CD4+ mean value 140.01 ± 84.16), whereas in LM the number of CD8+ cells was increased in comparison with CD4+ cells (approximately × 1.8 higher, CD8+ mean value 108.7 ± 12.8 *vs.* CD4+ mean value 60.49 ± 10.88, $p < 0.0001$).

When the T lymphocytes' populations were divided according to intra- or peritumoral location, we observed a significant prevalence of T cells in peritumoral over intratumoral tissue. In HCC the PT/IT ratio was 3.5 for CD8+ (PT mean value 231.70 ± 162.66 *vs.* IT mean value 65.3 ± 65.4) and 5.5 for CD4+ cells (PT mean value 237.05 ± 149.77 *vs.* IT mean value 43.10 ± 52.39); statistical analysis revealed significant differences for both CD4+ and CD8+ lymphocytes, intratumoral *vs.* peritumoral ($p < 0.0001$); no significant differences were found between CD4+ and CD8+ cells, when we compared their number in IT and PT areas, respectively (Figure 2A). In LM the ratios were 4.3 for CD8+ (PT mean value 176.00 ± 120.13 *vs.* IT mean value 41.10 ± 0.47) and 10.0 for CD4+ cells (PT mean value 109.9 ± 160.93 *vs.* IT mean value 11.00 ± 1.55); statistical analysis revealed significant differences between IT and PT area for both CD4+ and CD8+ cells ($p < 0.0001$), and also between CD4+ and CD8+ cells, in IT ($p < 0.0001$) and PT areas ($p < 0.005$) (Figure 2B).

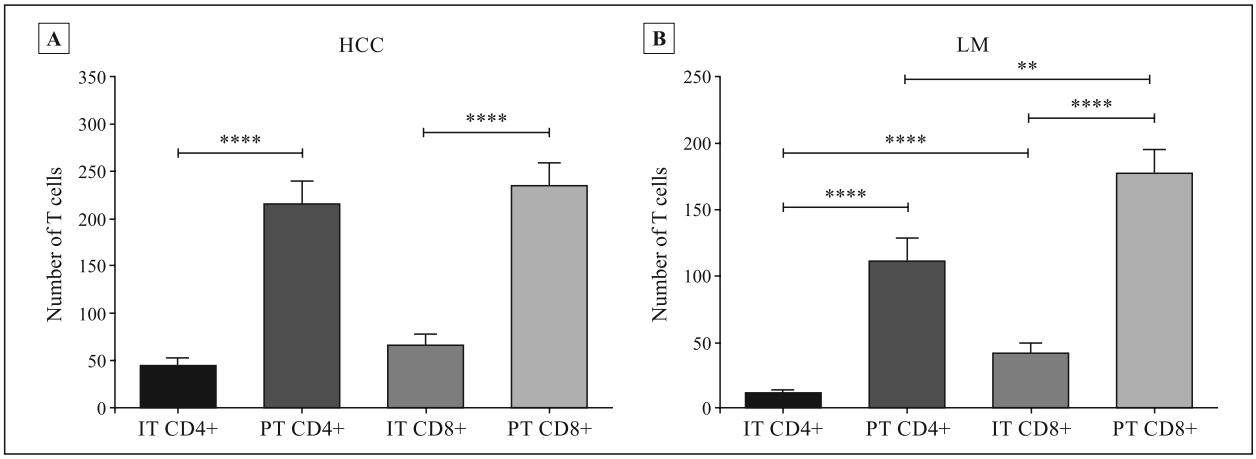

Figure 2. Comparison between number of intratumoral (IT) and peritumoral (PT) CD4+ and CD8+ lymphocytes in hepatocellular carcinoma (HCC, **A**) and liver metastases (LM, **B**). Bars and whiskers represent mean values ± SEM. $^{**}p < 0.005$, $^{****}p < 0.0001$; Mann-Whitney U test

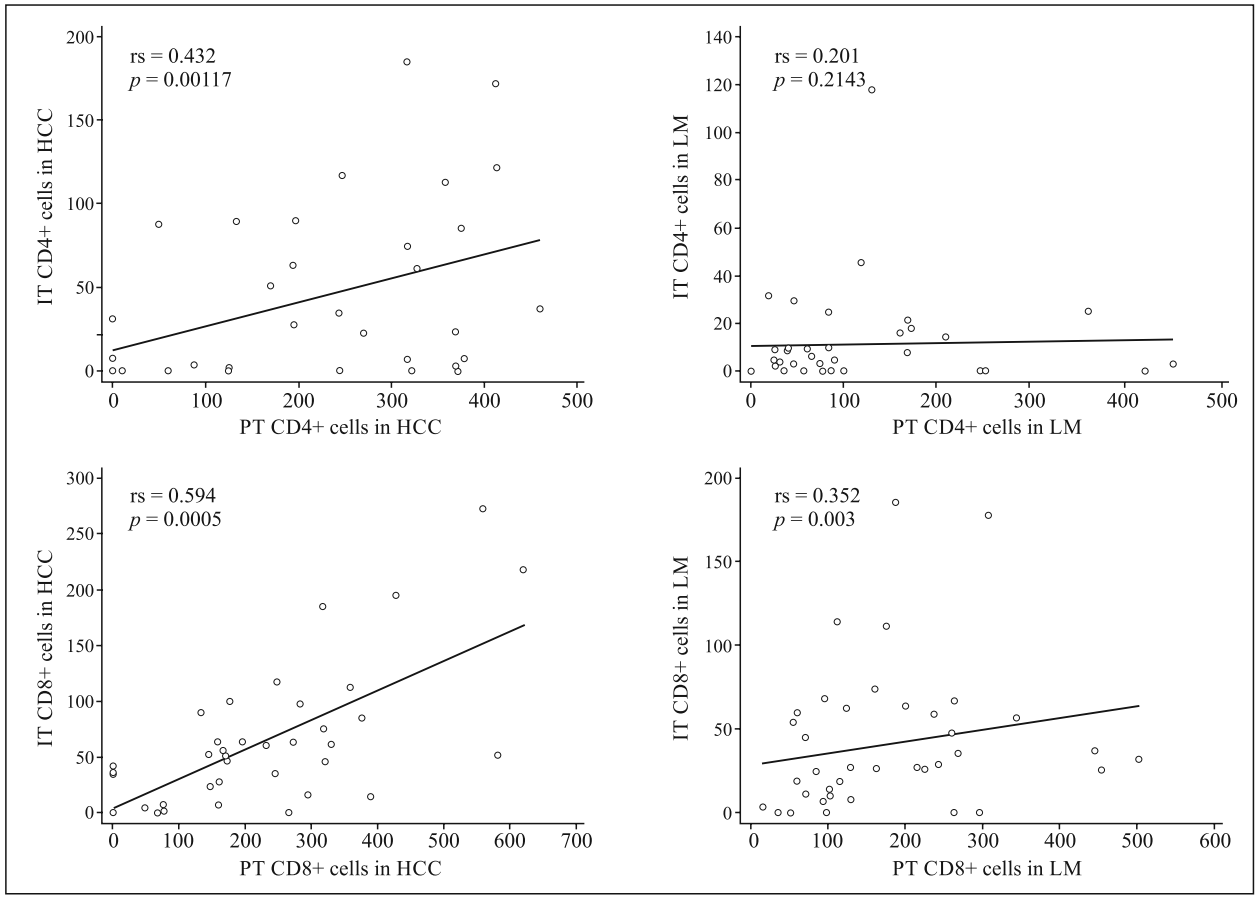

Figure 3. Correlation plots of peritumoral (PT) and intratumoral (IT) CD4+ and CD8+ lymphocytes' number in hepatocellular carcinoma (HCC) and liver metastases (LM). Spearman's correlation test

The correlation analysis between the number of lymphocytes in IT and PT areas revealed a medium positive association between either CD4+ or CD8+ cells in correspondence to HCC, whereas in LM the results indicated a weak relationship for both subtypes of T lymphocytes (Figure 3).

Table 2. Clinical data of patients with hepatocellular carcinoma or liver metastases in relation to the threshold number of CD4+ and CD8+ lymphocytes in intratumoral and peritumoral territories

Clinicopathological characteristics	CD4+ lymphocytes		CD8+ lymphocytes	
	Intratumoral territory	Peritumoral territory	Intratumoral territory	Peritumoral territory
	T ⇩ *vs.* ⇧	T ⇩ *vs.* ⇧	T ⇩ *vs.* ⇧	T ⇩ *vs.* ⇧
Hepatocellular carcinoma				
Age (years)				
< 63	13 *vs.* 3	6 *vs.* 10	14 *vs.* 2	10 *vs.* 6
	$p = 0.07$	$p = 0.5$	$\mathbf{p = 0.03}$	$p = 0.31$
≥ 63	9 *vs.* 10	10 *vs.* 9	10 *vs.* 9	8 *vs.* 11
Tumor stage				
I–II	13 *vs.* 6	8 *vs.* 11	13 *vs.* 6	8 *vs.* 11
	$p = 0.50$	$p = 0.73$	$p = 1$	$p = 0.31$
III–IV	9 *vs.* 7	8 *vs.* 8	11 *vs.* 5	10 *vs.* 6
Histological grade				
G1–G2	16 *vs.* 12	15 *vs.* 13	19 *vs.* 9	15 *vs.* 13
	$p = 0.21$	$p = 0.09$	$p = 1$	$p = 0.69$
G3	6 *vs.* 1	1 *vs.* 6	5 *vs.* 2	3 *vs.* 4
Liver metastases				
Age (years)				
≤ 68	12 *vs.* 6	8 *vs.* 10	11 *vs.* 7	12 *vs.* 6
	$p = 1$	$\mathbf{p = 0.02}$	$p = 1$	$p = 0.51$
> 68	15 *vs.* 6	17 *vs.* 4	13 *vs.* 8	11 *vs.* 10
Histological grade				
G1–G2	15 *vs.* 8	15 *vs.* 8	14 *vs.* 9	14 *vs.* 9
	$p = 0.72$	$p = 1$	$p = 1$	$p = 1$
G3–G4	12 *vs.* 4	10 *vs.* 6	10 *vs.* 6	9 *vs.* 7
Tumor extension				
One lobe	17 *vs.* 9	14 *vs.* 12	16 *vs.* 10	15 *vs.* 11
	$p = 0.71$	$\mathbf{p = 0.02}$	$p = 1$	$p = 1$
Many lobes	10 *vs.* 3	12 *vs.* 1	8 *vs.* 5	8 *vs.* 5

T — threshold (mean value/group), ⇩ — number of cases with CD4+/CD8+ lymphocytes mean value/case lower than threshold, ⇧ — number of cases with CD4+/CD8+ lymphocytes mean value/case higher than threshold; (Fisher's 2 × 2 test)

When the threshold values were applied for T cells' populations according to mean values (bars in Figures 2A, B), the association between the defined subgroups and clinical variables (Table 2) was tested; age > 63 was partially connected with decreased number of both populations of T lymphocytes. Moreover, we observed that decreased number of CD4+ cells within metastasized cancer islets was associated with increased number of lobes in LM.

Survival analysis

Based on Cox survival test and Kaplan-Meier plot we observed that the advanced stages (III and IV) in HCC and loss of histological differentiation (poor and undifferentiated grade) in LM are connected with shorter median OS rate (Figure 4). When we focused on T cells populations, the decreased number of CD8+ cells in area surrounding tumor in LM was associated with shorter OS (Figure 4). We also checked the PFS rates and no associations were found, neither based on clinical or morphological data.

Cox hazard test analysis revealed no significant relationship between variables and OS with the exception of HCC TNM staging. On the contrary, for LM we found that decreased number of peritumoral CD8+ lymphocytes may have independent prognostic value in LM cases with poorly or undifferentiated grade (Table 3). Again, for PFS data we found no connections with clinical and morphological findings.

Discussion

Despite the current knowledge on the complex behavior of TILs in the hepatic microenvironment [20, 38, 39], several issues are still in debate. It is unanimously accepted that the immune cells may inhibit the tumor growth and progression by malignant cells recognition and elimination, processes defined as immunesurveillance [40, 41]. If immunesurveillance

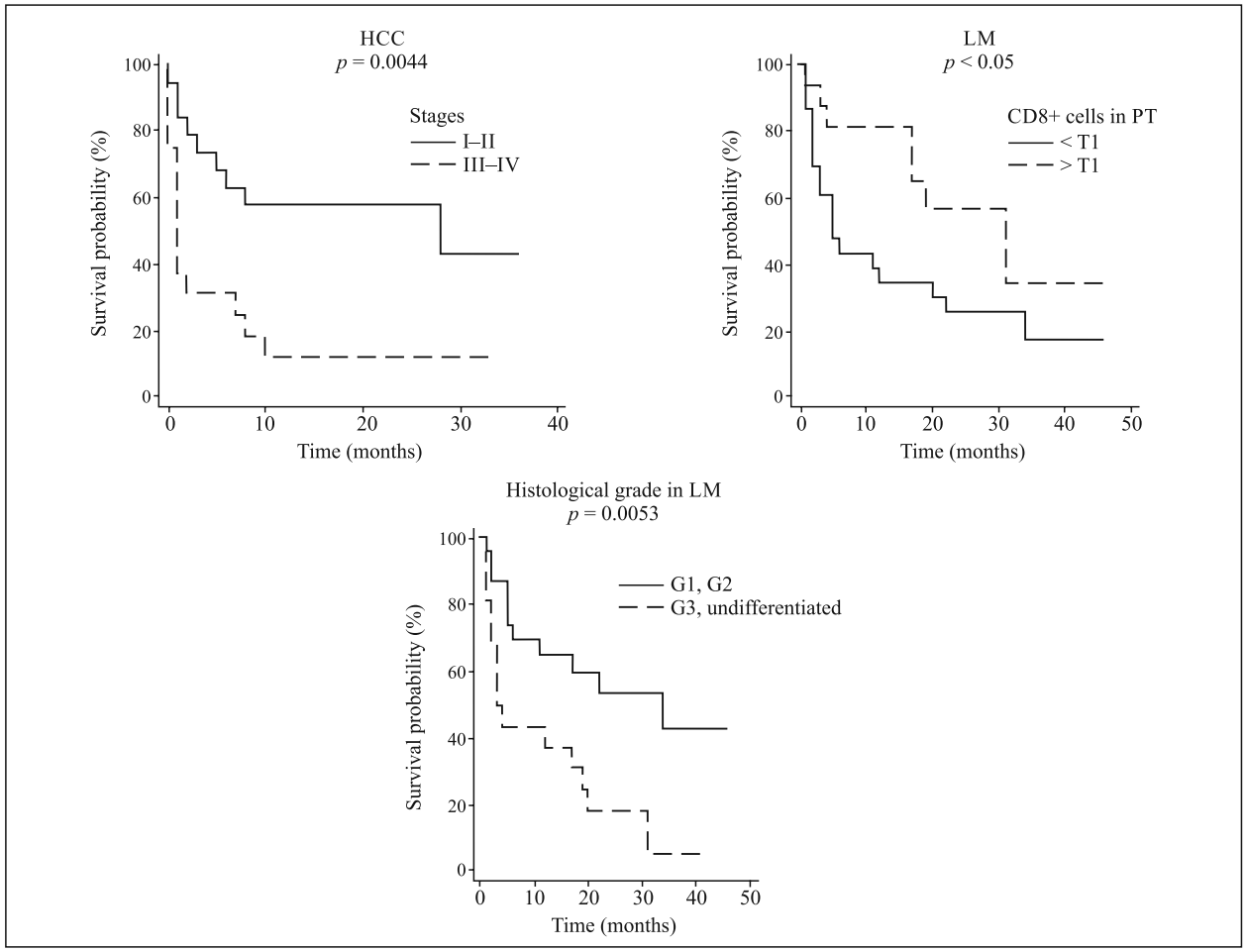

Figure 4. Kaplan-Meier curves show the prognostic value of tumor stage in hepatocellular carcinoma (HCC), and of peritumoral CD8+ lymphocytes and histological grade in liver metastases (LM)

Table 3. Selection of univariate and multivariate Cox regression analyses of overall survival rates in hepatocellular carcinoma and liver metastases

Parameters	Univariable analysis		Multivariable analysis	
	***p* value**	**Hazard ratio (95% CI)**	***p* value**	**Hazard ratio (95% CI)**
Hepatocellular carcinoma				
Tumor stage ≥ III *vs.* ≤ II	0.033	2.51 (1.07–5.86)		
Liver metastases				
Histological grade G3–4 *vs.* G1–2	0.009	2.82 (1.28–6.20)	0.006	2.98 (1.35–6.55)
Peritumoral CD8+ ⇩ *vs.* ⇧	0.049	0.45 (0.19–1.06)	0.047	0.42 (0.18–0.99)

Cox analysis of ⇧ vs. ⇩ means that the hazard ratio (HR) and *p* values are related to the occurrence of decreased number of T cells; only results with $p < 0.05$ were shown

fails, the tumor cells could be either chronically preserved or modified by the interactions with immune cells, generating new tumor variants able to escape the immune system by several mechanisms [40, 41]. Therefore, the immunodeficient status may facilitate the risk of tumor development — an event amplified

also by the tumor potential to generate an immunosuppressive microenvironment which prevents the antitumor immunity. The variability of the cellular and molecular cross-talk in tumor microenvironment (yet impossible to quantify) directly induces the variability of the antitumoral or/and protumoral capacity expression. As a consequence, the mechanisms which regulate TILs pro- or antitumoral activity have not been satisfactorily understood.

Within this context, we focused on the study of CD4+ and CD8+ T lymphocytes present in the hepatic microenvironment in two different conditions, namely HCC — as a primary tumor — and liver metastases. Our comparative analysis aimed to identify the similarities and differences between the lymphocytes' numbers in intra- and peritumoral tissues (including the relationship with survival) in the progression of primary and secondary tumor, respectively.

Despite the differences in the pathomechanisms of the primary or secondary neoplastic process, we noted a similar pattern of T lymphocytes' distribution in HCC and LM, characterized by a higher number of both major types of T cells in PT area, at the invasive front, and their lower number inside the tumor tissue. Such observation has been refined by the separate quantitative assessment of CD4+ and CD8+ lymphocytes and statistical analysis that outlined two important differences between HCC and LM.

Firstly, HCC presented a quite similar number of CD4+ and CD8+ lymphocytes, whereas in LM the number of CD8+ cells was approximately twice than CD4+ lymphocytes. These data obviously indicates a different behavior of CD4+ and CD8+ lymphocyte subpopulations in primary *versus* secondary liver tumors. Quantitative arguments which attest CD8+ prevalence in LM suggest a stronger antitumor defense reaction in comparison to HCC. A possible explanation of enhanced CD8+ cells number in LM than in HCC may be related to the increased tumor angiogenesis in metastases [42–44] and to the intrinsic relationship between proangiogenic factors and TILs [45, 46].

Secondly, we noted the significant decrease of both CD4+ and CD8+ cells within liver tumor as compared to its margin. Such observation supports the cross-talk between cancer and liver microenvironment [47] and the indirect tumor-T cell "fatal" relationship may develop at least in two ways. Fas-dependent induced apoptosis of activated T cells may be induced by tumoral cells expressing FasL. Such FasL-counterattack was found in non-small cell lung [48] and kidney cancer [49], but it is also characteristic for metastatic colorectal cancer (CRC) [50]. Since CRC was primary tumor site in most patients with liver metastases we suppose that this tumor-lymphocyte relation may have strong connections with decreased number of intratumoral T lymphocytes in LM in comparison to HCC.

Another possible mechanism related to the low number of CD8+ cells may be associated with the presence of B7-H1 signaling molecule on tumor-infiltrating macrophages (TAMs) [51]. It was noted that TAMs play important role in tumor development and progression in liver [47]. TAMs are lured and activated by cancer cells, which secrete either M-CSF (macrophage colony stimulating factor) [47] or/and CEA (carcinoembryonic antigen) [52]. Our previous report showed that number of TAMs in LM was associated with overall survival (OS) [53]. TAMs were found as a main source of B7-H1 signaling molecule, which connects to PD-1 death receptor on activated CD8+ cells and therefore induces their apoptosis [51]. Since the number of T cells is much higher in the peritumoral tissue, we suppose that the T-cell specific pro-apoptotic signals triggered by cancer cells play a main role in the decrease of number of T cells in liver tumors, regardless of cancer origin.

Furthermore, the important role of CD8+ cells in inhibition of tumor progression was proven by survival analysis, since decreased number of CD8+ lymphocytes was associated with shorter OS in patients with liver metastases. Our results supplement the reports by other authors that support the positive prognosis value of CD8+ T lymphocytes in LM [33–35].

Concerning the relationship between the lymphocytes and clinicopathological parameters, our data showed lack of correlations, except for the reduced number of CD8+/CD4+ lymphocytes and age over 63 or 68 years for HCC and LM respectively, and for the decreased number of intratumoral CD4+ lymphocytes and the increase of lobular extension in LM. These results reflect the difficulty to confirm the direct connection of lymphocytes to the clinical behavior of the tumors, either primary or secondary.

Finally, the main question that rises is related to the mechanisms that govern different antitumoral responses in primary and secondary tumors, respectively. Based on the above mentioned results, we can reiterate the intervention of the tumor FasL-counterattack or B7-H1 secretion.

Moreover, the absence of the correlation between TILs and survival in HCC can be interpreted as a consequence of an intense loss of lymphocytes through the proapoptotic processes that develop in the microenvironment of liver parenchyma. It is possible that in HCC these processes act on CD4+ and CD8+ T cells both in intra- and peritumoral tissue (and not only on IT lymphocytes as in LM). Such

possibility offers new alternative for understanding lymphocytes' role in primary and secondary tumor progression than the widely-held paradigm based on the intratumoral balance between regulatory and cytotoxic T lymphocytes [20, 38, 39] and the obligatory role of CD4+ T cells recruitment for the induction of an efficient antitumoral action of CD8+ T cells [35].

A special remark is compulsory to justify the methodology of our study that uses only the immunohistochemical assessment of the lymphocytic populations. At a glance, the absence of the advanced molecular biology techniques (*e.g.* cytometric analysis of tissue homogenates that can offer by *ex vivo* manipulation a better understanding of the *in vivo* cellular expression [54] could be considered as a major limitation for the obtained results. However, the morphological approach is much better suited for the identification and counting of CD4+ and CD8+ lymphocytes in their proper location in liver parenchyma, as shown by us for intra- and peritumoral territories. This approach made it possible to perform analysis of the correlation between the T lymphocytes' number and survival due to the use of the archived tumoral tissues.

Acknowledgements

Dr. Simona Eliza Giuşcă acknowledges financial support of the POSDRU/159/1.5/136893 project entitled "Strategic partnership for the improvement of the quality of research in medical universities through doctoral and postdoctoral scholarships — DocMed. Net_2.0", funded by the European Social Found, Human Resources Development Operational Programme 2007–2013.

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Submitted: 7 July, 2015
Accepted after reviews: 22 September, 2015
Available as AoP: 24 September, 2015