ORIGINAL PAPER

Immunohistochemical study of colorectal cancer liver metastases: the immune/inflammatory infiltrate

SIMONA ELIZA GIUŞCĂ[1)], FL. ELOAE ZUGUN[2)], E. TÂRCOVEANU[1)], E. CARASEVICI[2)], CORNELIA AMĂLINEI[3)], IRINA-DRAGA CĂRUNTU[3)]

[1)]*Department of Surgery, I[st] Surgery Clinic*
[2)]*Department of Immunology*
[3)]*Department of Histology*
"Grigore T. Popa" University of Medicine and Pharmacy, Iassy

Abstract

Our study is focused on the investigation of the immune/inflammatory infiltrate in liver metastases secondary to colorectal cancer. Twenty cases of colorectal liver metastases have been studied, including eight with recurrent metastases occurred after a previous treatment by thermonecrosis (group 1) and 12 with primary metastases treated exclusively by surgery (group 2). The cases were investigated by routine histopathological exam and by immunohistochemistry, using CD3, CD20 and CD68 antibodies. The design of the study envisages a comparative qualitative and quantitative evaluation of the B- and T-lymphocytes and macrophages inside the tumor and at the interface between liver parenchyma and tumor. Student's *t*-test was used for all statistical comparisons. The qualitative exam revealed, for both groups, the presence of an important T-lymphocyte, and respectively B-lymphocyte cell population at the interface between the tumor and the liver parenchyma, the number of intratumoral cells being extremely reduced. The statistical analysis showed significant differences ($p<0.05$ for groups 1 and 2, T- and B-lymphocytes, intratumoral *vs.* peritumoral). However, the comparison of group 1 with group 2 revealed no statistically significant differences between the mean value of intratumoral and peritumoral T- and B-lymphocytes, respectively. The qualitative exam revealed the presence of a well represented macrophage cell population, with a heterogenous distribution from case to case. This finding was confirmed by numerical information, with a lack of a statistically significant difference between the mean number of macrophages quantified intra and peritumoral, for both study groups. However, statistically significant differences were noticed between intratumoral and peritumoral mean value, respectively, for group 1 *vs.* group 2 ($p<0.05$). T-lymphocytes are the most numerous, their peritumoral location being the landmark for the histoarchitecture of the immune/inflammatory infiltrate and conducting the immune response developed at the interface between the tumor and liver parenchyma. The quantitative assessment of the immune infiltrate shows similar features in surgically resected metastases and recurrent metastatic disease after thermonecrosis. On the contrary, the quantitative evaluation of the macrophage population indicates a functional association rather with the primary metastasis process than with the recurrent metastatic disorder.

***Keywords*:** colorectal carcinoma, liver metastasis, immune/inflammatory infiltrate.

Introduction

As colorectal cancer (CRC) is the most frequent cause for liver metastases, present at more than 35% of the patients at the time of the primary tumor diagnosis and at over 25% after its surgical treatment [1]. The occurrence of liver metastases over a two years period after the primary resection [2], reported for 70–80% of the cases represents a negative prognosis factor, their existence if left untreated being associated with a 5% or less survival rate at five years [3]. However, after combined therapy, the patients can have excellent results, leading to a five-year overall survival rates of up to 58% [4].

Due to the raised incidence and tissue accessibility for biologic investigation, CRC and its metastases are probably the most studied malignancies, the researchers' contributions substantiating the information regarding pathogenesis, including the molecular and genetic areas. Consequently, the indications for hepatectomy in metastases secondary to CRC have been reevaluated and extended. Moreover, the evaluation of the prognosis is mandatory in order to orient the surgical and chemotherapy treatment plan according to the individual recurrence risk factor.

Starting from the characteristics of the carcinogenesis *vs.* metastatic process, although the role of the prognosis markers for CRC was recently revised [5–8], it is believed however that their generalization for resected liver metastases is nevertheless inadequate [9]. In the identification of the prognosis factors for colorectal liver metastases, we must take into consideration the necessary cellular adjustments for the acquirement of the metastatic competence, implying significant changes in tumor suppressor genes and oncogenes in their transition towards metastastic dissemination [10–13]. Furthermore, the cellular response in the liver regenerative environment is different after resection, as compared to the one existent after the resection of the primary tumor.

The clinicopathological prognosis factors include stage of the primary tumor, period of time between primary tumor resection and metastases diagnosis, number and dimensions of the metastases, preoperatory CEA level. Even though these factors can assess survival and recurrence risk after resection of metastases [14, 15], they do not possess the sensitivity needed in order to establish a precise individual prognosis. This statement is sustained by the fact that patients with similar clinical and pathological variables can develop different relapses [16]. Thus, the necessity of more sensitive prognosis factors occurred, defined by molecular features, based on specific tumoral biology aspects causing recurrence.

A number of markers, including proliferation indices, telomerase, thymidylate synthase and microvessel density have all shown strong evidence of prognostic utility and await prospective validation [9]. For other markers, such as 18q LOH, apoptotic regulators, p53, the prognostic value is still not completely established, mainly due to the incapacity of the research methods account for their biological complexity [9]. This is the reason they are called potential markers.

This study was developed departing from two premises. The first is represented by the current interest for the establishment of prognostic factors for colorectal liver metastases. The second is founded upon the role of the inflammatory/immune tumoral infiltrate investigated mainly at the level of the primary tumor, its behavior creating a Dr. Jekyll or Mr. Hyde enigma [17]. A close look at the literature reveals rather scarce data regarding immune/inflammatory infiltrate in the liver on the metastatic background.

Under these circumstances, our study is focused on the investigation of this infiltrate in liver metastases secondary to CRC, surgically removed and in recurrent liver metastases after thermonecrosis. The design of the study envisages a comparative evaluation of the B and T-lymphocytes and macrophages inside the tumor and at the interface between liver parenchyma and tumor. Our aim is to quantitatively characterize the immune and inflammatory component and to establish the presence or absence of statistically significant differences between primary liver metastases and recurrent metastatic disease.

Material and Methods

Twenty cases of colorectal liver metastases have been studied, including eight with recurrent metastases occurred after a previous treatment by thermonecrosis (group 1) and 12 with primary metastases treated exclusively by surgery (group 2). The cases were investigated by routine histopathological exam and by immunohistochemistry, using CD3, CD20 and CD68 antibodies.

Collected tissues were fixed for 24 hours in buffered formalin and processed for paraffin embedding. Serial sections of 4–5 μm were dewaxed and stained with Hematoxylin–Eosin, or performed for immunohistochemistry. After blocking the endogenous peroxidase and non-specific binding, the sections were incubated with one of the primary antibodies, ready-to-use: (i) anti-CD3 mouse monoclonal antibody (clone UCHT1, DAKO); (ii) anti-CD20 mouse monoclonal antibody (clone L26, DAKO); (iii) anti-CD68 mouse monoclonal antibody (clone kP1SC, DAKO). The immune reaction was amplified using the appropriate secondary antibody and the Streptavidin–Biotin–Peroxidase HRP complex (DAKO). Sections were then developed using 3,3'-diaminobenzidine tetrahydrocloride chromogen (DAKO), under microscope control. The sections were finally counterstained with Mayer's Hematoxylin. For each antibody, we also performed the negative control.

The quantitative evaluation was performed through the quantification of cells CD3, CD20 and CD68 positive, respectively. The mean value was obtained by analyzing 10 microscopic fields with the highest cellular density, at 400× magnification, separately for the intratumoral and peritumoral territories. Student's *t*-test was used for all statistical comparisons.

Results

Qualitative analysis

The analysis of the immune/inflammatory infiltrate revealed the following aspects:

- T-lymphocytes represented the predominating cell population, identified by immunohistochemistry in both study groups; they generate thick cell tapes or pseudofollicular structures at the boundary between the metastatic nodules and the remaining hepatic parenchyma (Figure 1) and penetrate through the tumoral components (Figure 2);
- B-lymphocytes were present in small number; intratumoral they were scattered (Figure 3); peritumoral, they were associated with the T lymphocytes, isolated (Figure 4) or in the pseudo-follicular structures;
- Macrophages represented an important constituent of the inflammatory infiltrate, located both peri- and intratumoral (Figures 5 and 6), disseminated among the lymphocytic component.

Quantitative analysis

- T-lymphocytes: for group 1, intratumoral mean value was 23.32±19.01, and peritumoral 55.15±32.83, with statistically significant differences intratumoral versus peritumoral (p=0.032); for group 2, intratumoral mean value was 19.01±8.97, and peritumoral 78.15±47.24, with statistically significant differences intratumoral *vs.* peritumoral (p=0.00032) (Figure 7);
- B-lymphocytes: for group 1, intratumoral mean value was 2.02±3.19, and peritumoral 19.95±14.21, with statistically significant differences intratumoral versus peritumoral (p=0.003); for group 2, intratumoral mean value was 2.75±1, and peritumoral 21.63±13.3, with statistically significant differences intratumoral versus peritumoral (p=0.0000) (Figure 8);
- Macrophages: for group 1, intratumoral mean value was 14.07±13.16, and peritumoral 20.75±13.68, without statistically significant differences intratumoral versus peritumoral; for group 2, intratumoral mean

value was 51.55±42.11, and peritumoral 60.86±42.4, without statistically significant differences intratumoral *vs*. peritumoral (p=0.0000) (Figure 9).

Tables 1 and 2, respectively, present the mean values with standard mean deviation, intratumoral and peritumoral for each case and immunohistochemical marker in groups 1 and 2, respectively.

Statistical evaluation of T-lymphocytes revealed the absence of any significant differences between groups 1 and 2, intratumoral and peritumoral (Figure 7). Similar results were obtained for B-lymphocytes (Figure 8). In exchange, statistical analysis for macrophage population showed significant differences between groups 1 and 2, intratumoral and peritumoral (Figure 9).

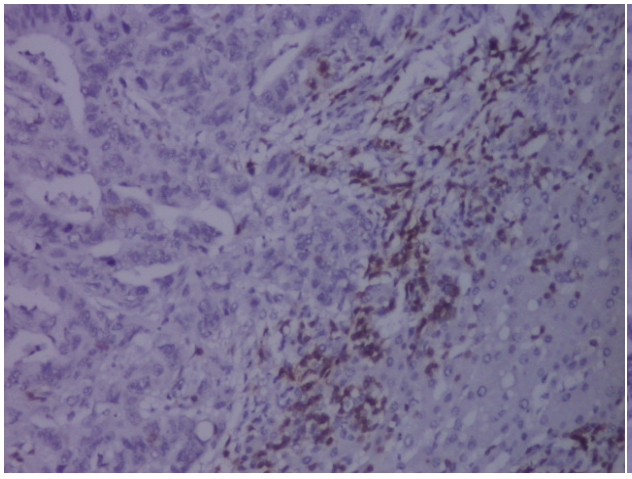

Figure 1 – ***CD3-positive T-lymphocytes, located peritumoral (IHC, anti-CD3, ob. ×10).***

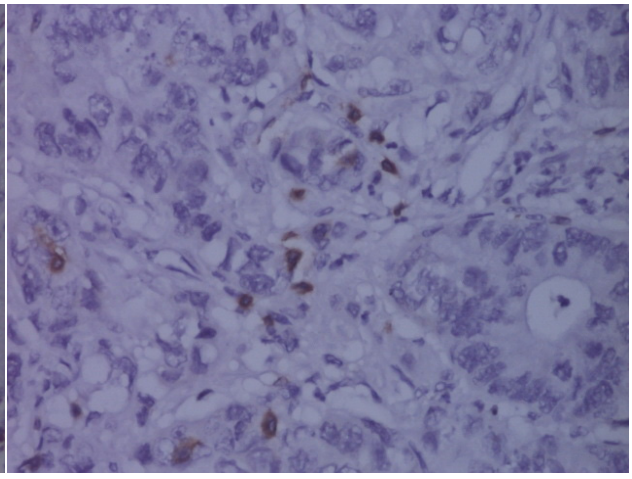

Figure 2 – ***CD3-positive T-lymphocytes, located intratumoral (IHC, anti-CD3, ob. ×20).***

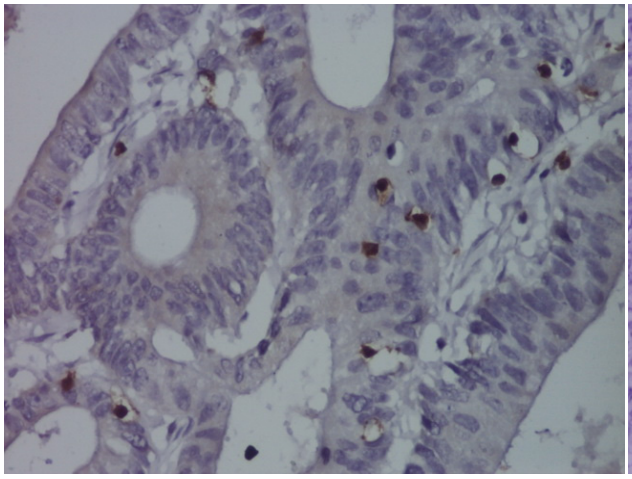

Figure 3 – ***CD20-positive B-lymphocytes, located intratumoral (IHC, anti-CD20, ob. ×20).***

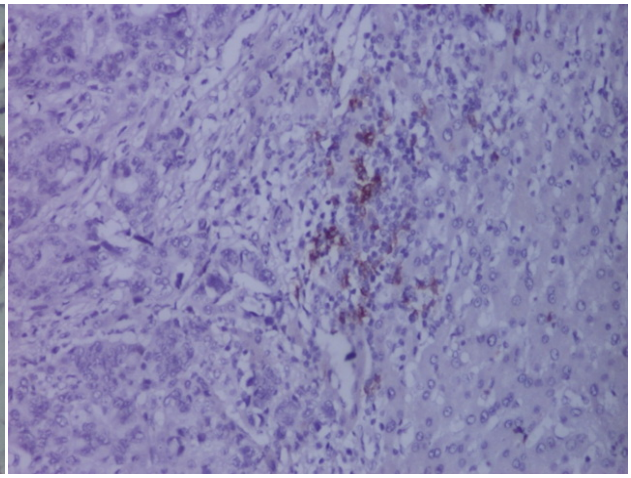

Figure 4 – ***CD20-positive B-lymphocytes, located peritumoral (IHC, anti-CD20, ob. ×10).***

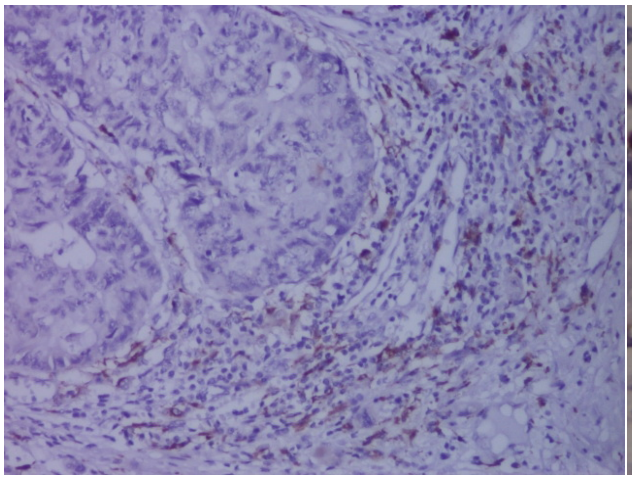

Figure 5 – ***CD68-positive macrophages, located peritumoral (IHC, anti-CD68, ob. ×10).***

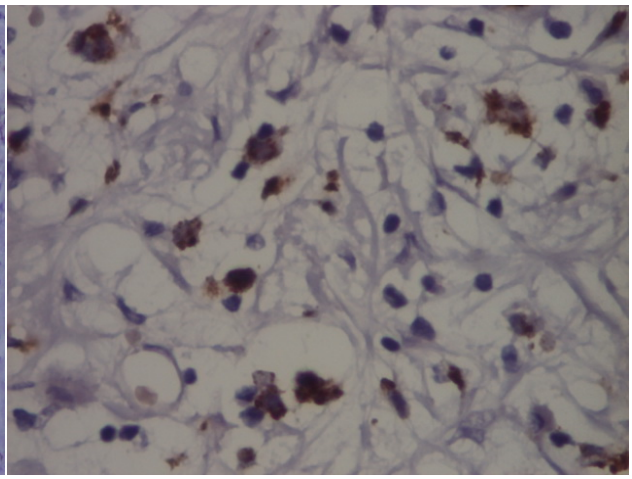

Figure 6 – ***CD68-positive macrophages, located intratumoral (IHC, anti-CD68, ob. ×40).***

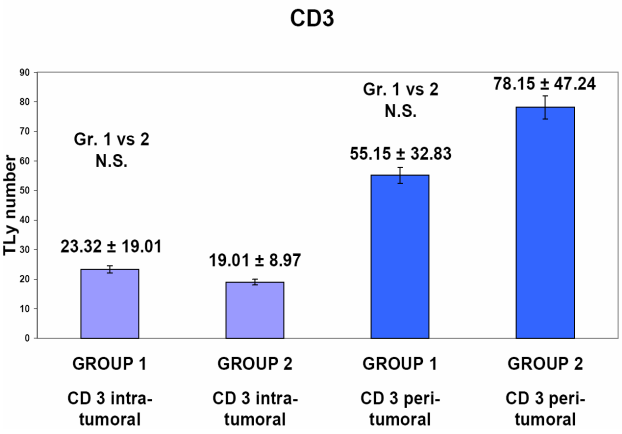

Figure 7 – *Assessment of T-lymphocyte cell population, peritumoral and intratumoral, group 1 vs. group 2.*

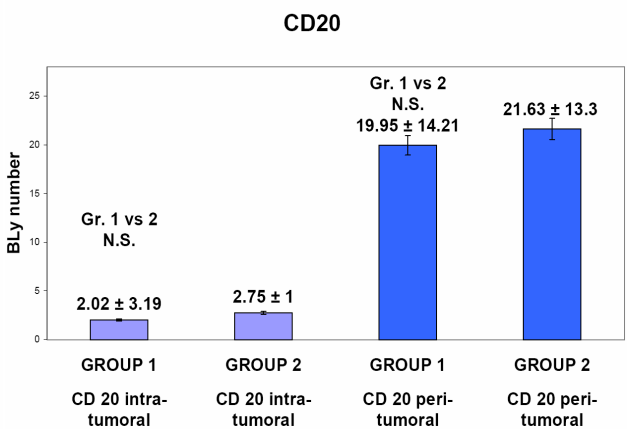

Figure 8 – *Assessment of B-lymphocyte cell population, peritumoral and intratumoral, group 1 vs. group 2.*

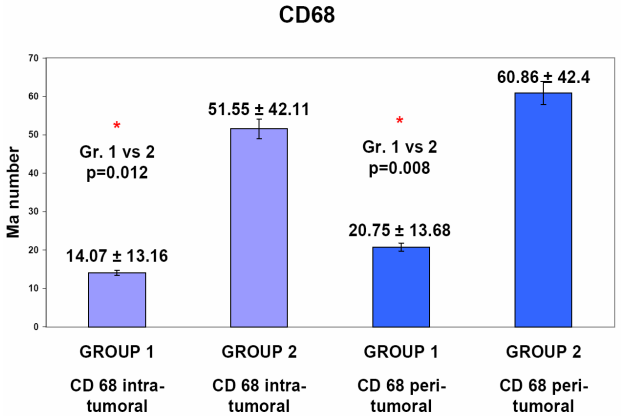

Figure 9 – *Assessment of macrophage population, peritumoral and intratumoral, group 1 vs. group 2.*

Table 1 – *Group 1. T-lymphocyte, B-lymphocyte, and respectively macrophage population: numerical data*

Case No.	CD3		CD20		CD68	
	Intra-tumoral	*Peri-tumoral*	*Intra-tumoral*	*Peri-tumoral*	*Intra-tumoral*	*Peri-tumoral*
1.	39.20±18.01	91.00±30.06	8.20±2.58	28.80±9.20	13.40±2.96	28.00±5.29
2.	55.00±10.27	49.20±23.97	0	1.60±1.50	31.40±12.70	18.00±4.47
3.	6.60±2.79	7.60±2.70	0	15.80±7.94	4.80±3.27	8.80±3.80
4.	0	16.60±8.84	0	16.80±7.12	0	12.40±2.50
5.	39.40±9.28	81.60±32.54	2.40±2.07	13.20±5.97	32.20±9.67	51.60±15.22
6.	13.60±3.84	57.80±20.24	0	21.60±9.15	22.40±9.30	18.20±8.28
7.	15.00±8.00	42.20±9.52	0	49.40±22.58	0	13.60±6.22
8.	17.80±9.12	95.20±22.20	5.60±2.40	12.40±7.30	8.40±3.43	15.40±8.38
TOTAL	23.32±19.01	55.15±32.83	2.02±3.19	19.95±14.21	14.07±13.16	20.75±13.68
p (intra- *vs.* peri-)	0.032		0.003		0.336	

Table 2 – *Group 2. T-lymphocyte, B-lymphocyte, and respectively macrophage population: numerical data*

Case No.	CD3		CD20		CD68	
	Intra-tumoral	*Peri-tumoral*	*Intra-tumoral*	*Peri-tumoral*	*Intra-tumoral*	*Peri-tumoral*
1.	6.00±2.54	141.80±46.01	3.40±1.67	42.20±13.42	40.00±14.01	59.60±23.26
2.	15.60±3.43	173.20±28.22	1.00±1.00	47.40±15.32	170.20 ±54.47	53.40±17.74
3.	3.80±2.58	13.80±4.14	2.40±1.51	7.40±6.34	77.00±10.75	11.80±34.80
4.	24.20±11.88	102.20±40.18	3.00±2.91	12.40±4.77	71.00±35.02	131.40±41.65
5.	28.20±12.27	110.40±29.48	2.20±0.83	28.20±14.39	60.20±16.45	134.00±54.12
6.	15.20±6.53	63.40±19.32	4.80±3.56	34.60±15.82	27.40±9.23	41.40±13.04
7.	22.40±7.98	87.60±36.77	2.40±2.30	12.40±6.22	26.80±10.00	44.20±8.98
8.	9.20±6.09	28.60±9.44	2.00±0.70	12.40±6.10	18.80±5.80	32.20±15.60

Case No.	CD3		CD20		CD68	
	Intra-tumoral	*Peri-tumoral*	*Intra-tumoral*	*Peri-tumoral*	*Intra-tumoral*	*Peri-tumoral*
9.	30.80±15.08	40.40±18.67	3.20±1.30	19.20±11.23	21.80±7.39	21.20±7.72
10.	27.00±8.63	42.80±16.61	3.80±1.78	18.80±9.75	25.20±7.69	47.00±8.00
11.	21.00±9.79	64.60±22.45	1.80±1.92	11.20±4.65	46.80±14.70	117.20±47.45
12.	24.80±10.96	69.00±19.64	3.00±2.54	13.40±8.20	33.40±11.28	37.00±22.72
TOTAL	19.01±8.97	78.15±47.24	2.75±1.00	21.63±13.3	51.55±42.11	60.86±42.40
p (intra- *vs.* peri-)	0.00032		0.00000		0.59	

Discussion

In the extended studies dedicated to CRC, one of the main directions is represented by the identification and confirmation of the prognostic factors associated with survival. In this context, we can find the presence of the immune/inflammatory infiltrate that includes lymphocytes, neutrophils and macrophages; consequently, a special category of lymphocytes is defined – tumor-infiltrating lymphocytes (TILs) [18–21]. This subject raises many problems in view of its complex functional signification, which must be understood beyond the apparently simple histological picture – still image of molecular/biochemical interactions between normal cells, tumoral cells and matrix. Moreover, the immune/inflammatory infiltrate, together with fibroblasts and endothelial cells is integrated in the concept of tumoral microenvironment [22], whose importance increases due to the adaptive abilities influencing both primary tumor and correspondent metastases.

The biologic profile of liver metastases secondary to CRC is however different as compared to the primary tumor, their morphologic and functional background being incompletely defined. In the attempt to contribute to this characterization, our research was concentrated on the immune/inflammatory infiltrate, mainly through its intervention ability in the immune relationship established between metastasis and liver parenchyma. The liver is considered a singular immune organ [23, 24], dissemination at this level having a tolerant potential due to the action of a wide range of factors, among which the ability to favor development of regulator T-lymphocytes was recently described [25]. It is also stated that the immune system might protect the host against tumor development through immunosurveillance mechanisms [26].

Our research is individual by (i) the characteristics of the investigated groups and (ii) the precise quantification of intra- and peritumoral T- and B-lymphocytes and macrophages (and not by semi-quantitative scoring).

Recurrent metastatic disease after thermonecrosis *vs.* surgically resected metastases

Group 1 was formed by cases with recurrent liver metastases secondary to CRC. More precisely, these metastases were developed at a distance from a previous thermonecrosis ablation, performed as a treatment alternative when surgery was excluded as treatment option. Taking into account the biological and time framework of their occurrence, the lesions were defined as recurrent metastatic disease. The second group was formed by cases with liver metastases secondary to CRC with surgical indication and, consequently, resected. Taking into account the biological and time framework of their occurrence, the lesions were defined as *per primam* metastases. In the context of these evolution features, we started from the assumption that group 1, characterized by a more aggressive clinical evolution should have an immune/inflammatory infiltrate profile different from group 2. This assumption was not completely confirmed.

Lymphocytic population

Qualitative immunocytochemical exam revealed, for both groups, the presence of an important T-lymphocyte, and respectively, B-lymphocyte cell population at the interface between the tumor and the liver parenchyma, the number of intratumoral cells being extremely reduced. Particular aspects were noted at intratumoral level for four cases in group 1, where B-lymphocytes were absent, as well as a case where both types of lymphocytes were absent (the correctness of the immunocytochemical reactions being sustained by the positive control). The statistical analysis confirms the morphologic image, by significant differences ($p<0.05$ for groups 1 and 2, T- and B-lymphocytes, intratumoral *vs.* peritumoral).

The comparison of the results obtained from of the two study groups brings supplementary arguments for the understanding of the immune infiltrate action mechanism. At a first glance, from the clinical point of view, the recurrent metastases included in group 1 were considered, as we mentioned before, as lesions with a more severe biologic profile and evolution potential. Nevertheless, when we compared group 1 with group 2, the quantitative assessment indicated the fact that there were no statistically significant differences between the mean value of intratumoral and peritumoral T-lymphocytes, respectively. The absence of statistically signifycant differences was recorded at intratumoral and peritumoral level also for the mean value of B-lymphocytes.

The quality of independent prognostic factor for the immune/inflammatory infiltrate in CRC was investigated beginning from the '80s [18–21, 27–31]. Much less, research is dedicated to this infiltrate within the framework of liver metastases secondary to CRC. The main issues – still unanswered – concern the concrete modality by which the tumoral microenvironment intervenes in the metastatic process and the actual contribution of the innate and adaptive immune system [32–34]. Following this direction, the involvement of

effector memory CD8+ T-cells in the early metastatic stage of carcinogenesis is supported by Pagès F *et al.*, 2005 [35], who demonstrate that the massive presence of this cellular subtype in the infiltrate prevents metastasis by immune-mediated control.

According to the data from the literature [33–35], our study confirms the preponderance of T-lymphocytes as well as their massive peritumoral location. However, our study brings, by the double qualitative and quantitative evaluation, a support in favor of the hypothesis, which states that the immune infiltrate located at the tumoral-parenchyma interface indicates its involvement in the development of metastases through a cell-mediated immune response ensuring mainly immunotolerance [33]. Thus, the role of the immune infiltrate (mainly peritumoral) in the progress of the metastases becomes fairly obvious which leads us to the reevaluation of its significance as positive prognostic factor. Certainly, T-lymphocytes are the most important cellular components. Therefore, from the point of view of the lymphocytic "charge", our study reveals that surgically resected metastases do not differ from recurrent metastatic disease after thermonecrosis, which suggests that liver metastases (regardless of their stage) develop without inducing noticeable phenomena of immune rejection. Hence, we must stress the fact that our preliminary results do not overlap with the classic theory that considers the presence of T-lymphocytes as a positive prognosis factor. In order to bring solid evidence in support of the new hypothesis stating that T-lymphocytes predict a negative outcome, our future research direction intends to correlate this data with patients' survival.

Macrophage population

The immunohistochemical exam revealed the presence of a macrophage cell population, well represented for group 1 and very well represented for group 2, with a heterogenous distribution from case to case. This qualitative statement was confirmed by the numerical information, where we can notice that although the absolute numeric values are bigger for group 2 as compared to group 1, in both groups there were cases with more macrophages intratumoral and cases with more macrophages peritumoral. Statistical analysis indicated the lack of a significant difference between the mean number of macrophages quantified intra and peritumoral, for both study groups. Our results support their role (less well studied) in the metastatic process, by the production of factors, which may enhance angiogenesis, increased tumor growth, and invasion, as well as immune escape [33, 36–38].

In the metastatic process, the aggressive pro-tumorigenic/metastatic profile implies a molecular dialogue the tumoral cells, T-lymphocytes and macrophages. In the two types of metastasis analyzed, the macrophages follow a distinct quantitative scenario than T- and B-lymphocytes. Statistical analysis registered significant differences between intratumoral and peritumoral mean value, respectively, for group 1 *vs.* group 2 (p=0.012, p=0.008, respectively). This suggests an active participation of macrophages in the formation of *per primam* metastases, their functional potential being partially spent in recurrent metastatic disease.

Conclusions

Our study envisaged the qualitative and quantitative characterization of the T-, B-lymphocyte and macrophage cell population in the distinct framework of surgically resected metastases and that of recurrent metastatic disease after thermonecrosis. T-lymphocytes are the most numerous, their peritumoral location being the landmark for the histoarchitecture of the immune/inflammatory infiltrate and conducting the immune response developed at the interface between the tumor and liver parenchyma. The quantitative assessment of the immune infiltrate shows similar features in surgically resected metastases and recurrent metastatic disease after thermonecrosis. On the contrary, the quantitative evaluation of the macrophage population indicates a functional association rather with the primary metastasis process than with the recurrent metastatic disorder.

There still are a lot of questions and uncertainties about the relationship between immune/inflammatory infiltrate and liver metastasis. The positive prognostic role of T-lymphocytes is discussed, a new hypothesis indicating a negative role. Clearly, the issue represents an open research direction, which must be mandatorily completed by the development of multiple correlations, including with the survival rate.

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Corresponding author
Irina-Draga Căruntu, Professor, MD, PhD, Department of Histology, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, Iassy, 16 University Street, 700115 Iassy, Romania, e-mail: irina_caruntu@yahoo.com

Received: July 15th, 2009

Accepted: January 15th, 2010