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Corelation of Lymphocytic Infiltrates with the Prognosis of Recurrent Colo-Rectal Cancer

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Rezumat

Corelația infiltratelor limfocitare cu prognosticul recidivei cancerului colo-rectal

Introducere: Din ce în ce mai multe studii încearcă găsirea de factori de prognostic complementari stadializării TNM, iar studiul infiltratelor inflamatorii pare a fi destul de promițător în acest sens.
Material și metodă: Studiul reprezintă o analiză retrospectivă a 23 de piese de rezecție (tumori primare) aparținând pacienților care s-au prezentat in clinica noastra pentru recidive după cancer colo-rectal, pe o perioadă de 2 ani. Pentru fiecare pacient gradul de infiltrare cu celule cd3+, cd4+, cd8+, cd45ro și cd68+, la nivel peritumoral, la nivelul frontului de invazie și intratumoral a fost evaluat, folosind densitatea de celule pozitive și procentul de celule pozitive.
Rezultate: O densitate mică de celule cd8+ la nivelul țesutului normal peritumoral a fost asociată in cadrul studiului nostru cu un interval liber de boală lung (p=0,009), iar analiza de supraviețuire a demonstrat că abundența infiltratului cu celule cd8+ poate stratifica pacienții în funcție de riscul de recidivă (p=0,006). Infiltratele de la nivelul frontului de invazie și de la nivel intratumoral nu au fost în niciun fel corelate cu intervalul liber de boală.
Concluzii: Prezența de celule T-citotoxice la nivelul țesutului normal peritumoral poate fi asociată cu o tumoră agresivă din punct de vedere al riscului de recidivă.

Cuvinte cheie: prognosticul recidivei, cancer de colon și de rect, evaluare cantitativă a marcajului imunohistochimic, analiză digitală de imagine, celule T citotoxice

Abstract

Background: Recent studies are focusing on complementary prognostic and predictive markers that could complete the predictive TNM staging and one of the most promising directions is the study of tumor immune infiltrates.
Materials and methods: Our 2-year retrospective study includes resection specimens from the primary tumors of 23 patients presenting to our clinic for a local or a distant relapse after colon or rectal cancer. From every primary tumor specimen we obtained immunohistochemically stained slides in order to assess cd3, cd4, cd8, cd45ro and cd68 infiltrates. Digital analysis assessed the density and percentage of positively stained cells in the normal peritumoral tissue, invasive margin and center of the tumor.
Results: A small density of cd8 positive cells in the peritumoral region was strongly correlated with a longer disease-free interval (p=0.009) and the Kaplan-Meier survival analysis showed that the percentage of cd8+ T cells could be used to

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stratify patients in terms of relapse risk (p=0.006). We found no correlation between invasion front infiltrates and intratumoral infiltrates and the disease-free interval.
Conclusion: Our study concludes that cytotoxic T-cell infiltrates in the normal peritumoral tissue could be used to predict a more aggressive tumor in terms of the relapse risk.

Key words: relapse prognosis, colo-rectal cancer, quantitative assessment of immunohistochemical staining, digital image analysis, cytotoxic T cells

Introduction

For most solid tumors, the pathology TNM staging is decisive in choosing the therapeutic approach for each colo-rectal cancer patient. In terms of assessing the relapse prognosis the pN criterion system is one of the most important, however more and more studies have shown that some of the node-negative patients develop a relapse (1-4) and this is why current research is focused on finding complementary predictive and prognostic markers.

The main directions of study consist of lymph node statusassessment (5-12), molecular markers (13-19), imaging related prognostic scores (20, 21), pathology findings like perineural invasion, tumor emboli and/or necrosis (22) and more and more frequently inflammatory infiltrates (23-28). Interestingly, the first studies about the possible implications of the immune system in the progression or the control of cancer come from as early as 1863 when Rudolf Virchow discovered the presence of leucocytes in cancer tissue (23). Unfortunately though, interest towards studying immune infiltrates as prognostic markers has only recently developed.

Materials and Methods

The present study was designed as a retrospective study over a period of 2 years, from September 2009 to September 2011. The Ethical Committee of the "Grigore T. Popa" University of Medicine and Pharmacy approved the study and a signed informed consent form was obtained from every patient.

A total number of 23 patients were selected to take part in the study following their presentation to the First Surgical Clinic of the "St. Spiridon" University Clinical Hospital for a local relapse or for distant metastasis after a curative procedure for colonic or rectal adenocarcinomas. For the patients to be included, recurrence had to beconfirmed either by computer tomography or magnetic resonance imaging or by histological confirmation on a biopsy sample or an excised tumor specimen.

During this period a total of 91 patients with metastases or local recurrences after having been operated on for colorectal cancer presented to our clinic, but 68 patients (74.72 %) were excluded because of one of the following exclusion criteria:

- presence of a macroscopically visible metastasis at the time of the first surgery;
- neoadjuvant radiotherapy and/or neoadjuvant chemo-therapy, because of the possible alteration of the tumor microenvironment due to the presence of necrosis induced by the neoadjuvant therapy (28);
- previous neoplastic disease, other than the colorectal cancer that is suspected to be responsible for the recurrence, because of the possible alteration of the immune response directed against the tumor (28);
- patients who underwent the first procedure in another clinic and thus rendered us unable to access complete medical records and/or the paraffin-embedded tissue blocks from the time of the first procedure.

Medical records were inspected searching for sex, age of the patient at the time of the first surgical procedure for colorectal cancer, topography of the tumor, type of recurrence – local vs. distant. The disease free interval was calculated based on the data collected from the patient's medical recordsand used as an indicator of the aggressiveness of the tumor. From the pathology records we collected data such as the TNM staging, the dimensions of the tumor, the distance between the tumor and the margin of resection, the presence of tumor perforation and/or a peritumoral abscess, as well as the degree in which the lumen was obstructed, the infiltration of the serosa, the presence of perineural invasion and tumor emboli, the abundance of necrosis, the presence of a mucinous component, the degree of tumor differentiation (well, moderately and poorly differentiated), the number of harvested lymph nodes and the number of invaded lymph nodes.

From each tissue block we obtained one section that was 4 μm thick for standard haematoxylin-eosin staining and 5 sections with a 2 μm thickness on which we performed immunohistochemistry. *Table 1* presents the markers that were used and their concentrations. The working dilutions for the monoclonal antibodies are also found in *Table 1* – they were selected after a large number of trials to assess the best quality of the staining.

The stained slides were then acquired using a TissueGnostics system connected to a Zeiss Observer Z1 microscope (TissueGnostics GmbH, Austria) and the acquired images were then processed using the HistoQuest software (TissueGnostics GmbH, Austria).

On each slide we selected 9 regions of interest with the same surface, as follows: 3 regions of interest for the normal peritumoral tissue (*Fig. 1*), 3 regions of interest for the invasion front (*Fig. 2*) and 3 regions of interest for the intratumoral tissue (*Fig. 3*).

For each region of interest the cells were automatically counted using the HistoQuest software and the threshold distinguishing between positively marked cells was established using the internal algorithm provided by the software, which splits the cell population into 2 classes based on the intensity of the colour of the marker.

Statistical analysis was carried out using SPSS for Windows, version 17. Spearman's rank correlation test was used to evaluate correlations between different variables and

Table 1. *Monoclonal antibodies used for immunohistochemical staining*

Antibody	Type of the antibody and producer	Dilution	Cellular staining pattern	Type of cell targeted by the antibody
Anti-CD3	rabbit monoclonal/Neomarkers, California, USA	1:300	membrane	T cells in general
Anti-CD4	mouse monoclonal/Neomarkers, California, USA	1:20	membrane	T helper lymphocytes
Anti-CD8	mouse monoclonal/DAKO, Denmark	R.T.U	membrane	cytotoxic t cells
Anti-CD45ro	mouse monoclonal/Neomarkers, California, USA	R.T.U	membrane	memory T cells
Anti-CD68	mouse monoclonal, DAKO, Denmark	R.T.U	cytoplasmic, granular/diffuse	macrophages

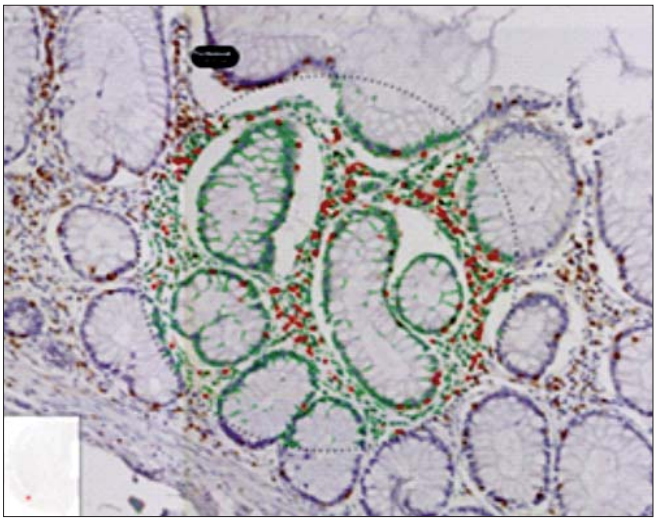

Figure 1. *Processed region of interest from the normal peritumoral tissue*

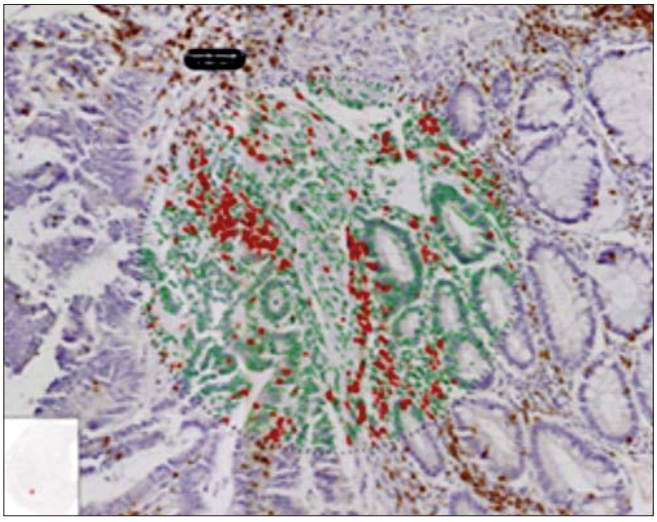

Figure 2. *Processed region of interest from the invasion front*

the disease free interval (considered as a continuous variable). We considered the results as being statistically significant at a level of statistical signification of 0.05. Kaplan-Meier survival analysis for disease-free survival was also performed and the log-rank test was used to search for statistically significant differences between the survival curves.

Results

From the 23 patients selected to take part in the study 52.2% (n=12) presented with a metastasis and 47.8% (n=11) presented with a local relapse. The mean age of the patients was 62.39±2.82 years with a minimum of 36 years and a maximum of 83 years. The mean time to relapse in our study was 21.15±1.99 months with a minimum of 7.8 months and a maximum of 38.3 months.

From a point of view of the topography of the original tumor 17.4% (n=4) of the patients had tumors which we classified as being located on the right colon (cecum, ascending colon, right colic flexure and transverse colon), 47.8% (n=11) of the patients had left colonic tumors (left colonic flexure, descending colon and sigmoid colon) and 34.8% (n=8) of the patients had recto-sigmoid and rectal tumors.The mean size of the tumor was 50.74±3.81 mm with a minimum of 10 mm and a maximum of 85 mm. A more detailed description of the morphoclinical characteristics of the study group can be found in *Table 2*.

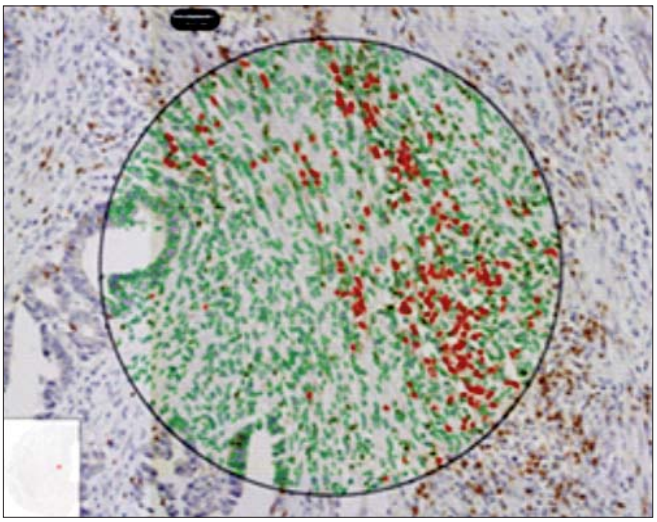

Figure 3. *Processed region of interest from the intratumoral tissue*

The mean hospital stay was 15.09±1.06 days, with a minimum of 8 days and a maximum of 27 days.

The following step was the analysis of correlations and

Table 2. *Morphoclinical characteristics of the study group*

		Frequency	Percentage
Sex	Female	10	43.5
	Male	13	56.5
pT	2	2	8.7
	3	15	65.2
	4	6	26.1
pN	0	1	4.3
	1	5	21.7
	2	5	21.7
	x	12	52.2
Differentiation	High degree of differentiation	2	8.7
	Moderate degree of differentiation	19	82.6
	Low degree of differentiation	2	8.7
Topography of the primary tumor	Right colon	4	17.4
	Left colon	11	47.8
	Rectum	8	34.8
Abundance of necrosis	Low	12	52.2
	Moderate	2	8.7
	High	9	39.1
Presence of vascular invasion	Absent	21	91.3
	Present	2	8.7
Tumor emboli in lymph vessels	Absent	13	56.5
	Present	10	43.5
Perineural invasion			
	Absent	18	78.3
	Present	5	21.7
Presence of a colloid component	Absent	18	78.3
	Present	5	21.7
Presence of a peritumoral abscess	Absent	21	91.3
	Present	2	8.7
Presence of a perforated tumor	Absent	22	95.7
	Present	1	4.3
Stenotic tumor			
	No	18	78.3
	Yes	3	13
Serosal layer macroscopically affected	No	8	34.8
	Yes	15	65.2
Presence of anemia	No	16	69.6
	Yes	7	30.4
Leucocytosis	Absent	13	56.5
	Present	10	43.5
Type of relapse	Metastasis	12	52.2
	Local relapse	11	47.8

correlations between commonly investigated morphoclinical factors and the disease free interval were the first to be investigated - the results can be seen in *Table 3*.

Table 3. *Results of the Spearman's rank correlation test between morphological characteristics of the primary tumor and the time to recurrence*

	Relapse free interval	
	rho	**p-value**
pTumor	0.022	0.924
Stenotic tumor	0.514	0.009
Perforated tumor	0.136	0.516
Resection margin (continuous variable)	-0.111	0.599
Infiltrated serosa	0.069	0.742
Acute inflammation	0.123	0.558
Chronic inflammation	-0.085	0.685
Peritumoral abscess	-0.104	0.621
Degree of tumor differentiation	0.309	0.133
Mucinous component	0.166	0.426
Perineural invasion	0.305	0.138
Intravascular tumor emboli	0.035	0.869
Necrosis	0.238	0.252

We then processed the slides and obtained quantitative information about the immunohistochemical staining by using the HistoQuest software according to the method described above. For each marker we calculated a mean of the density of positive cells and a mean of the percentage of positive cells for the intratumoral tissue, for the invasive margin and for the normal peritumoral tissue. The results of this quantitative analysis can be seen in *Table 4*.

Finally we performed a correlation analysis between the relapse free interval and the variables resulted from the quantitative analysis of the immunohistochemically stained slides. We found no correlation between the relapse free interval and the inflammatory infiltrate in the invasive margin and the intratumoral tissue, however we did find a strong correlation between a small percentage of cd8+ cells in the normal peritumoral tissue and longer disease-free survival ($p=0.009$), but no correlation between the density of cd8+ cells in the same region and the relapse-free interval was found. We also found a marginal correlation ($p=0.040$) between a low cd45ro+ cell density in the normal peritumoral tissue and a longer disease-free survival.

The Kaplan-Meier survival analysis (*Fig. 4*) further showed that the percentage of cd8+ T cells in the normal peritumoral tissue can be used to stratify patients in terms of disease-free survival ($p=0.006$ for the Log-Rank test). The groups were defined based on the median value of the percentage of cd8+ cells.

Discussion

In operated colo-rectal cancer patients relapse can usually be predicted by assessing the lymph node status, but in about 25% to 30% of the patients who are node-negative recurrence of the neoplastic disease can still occur (1,2). This is the reason why a lot of the studies involving colo-rectal carcinoma patients are more recently directed towards finding new prognostic and predictive markers that could be complementary to the

Table 4. *Results of the quantitative analysis of the immunohistochemically stained slides*

			Mean	Std. Deviation
Intratumoral	cd3	Density of positive cells (cells/square mm)	1919.55	2391.22
		Percentage of positive cells	21.17	27.10
	cd4	Density of positive cells (cells/square mm)	2101.37	1768.80
		Percentage of positive cells	24.46	20.89
	cd8	Density of positive cells (cells/square mm)	603.13	602.55
		Percentage of positive cells	6.55	6.52
	cd45ro	Density of positive cells (cells/square mm)	2363.38	1150.57
		Percentage of positive cells	25.26	11.07
	cd68	Density of positive cells (cells/square mm)	1755.02	1328.30
		Percentage of positive cells	19.35	12.21
Invasive front	cd3	Density of positive cells (cells/square mm)	2434.88	2132.14
		Percentage of positive cells	26.30	25.04
	cd4	Density of positive cells (cells/square mm)	1714.14	1493.32
		Percentage of positive cells	17.59	15.08
	cd8	Density of positive cells (cells/square mm)	882.86	1041.14
		Percentage of positive cells	8.66	10.35
	cd45ro	Density of positive cells (cells/square mm)	3018.68	1400.81
		Percentage of positive cells	30.89	12.70
	cd68	Density of positive cells (cells/square mm)	1939.25	1470.45
		Percentage of positive cells	19.92	14.05
Peritumoral normal tissue	cd3	Density of positive cells (cells/square mm)	1758.28	1998.42
		Percentage of positive cells	21.37	25.25
	cd4	Density of positive cells (cells/square mm)	2026.84	1717.31
		Percentage of positive cells	24.17	19.75
	cd8	Density of positive cells (cells/square mm)	676.78	760.27
		Percentage of positive cells	8.65	7.86
	cd45ro	Density of positive cells (cells/square mm)	1821.11	1199.53
		Percentage of positive cells	21.61	11.06
	cd68	Density of positive cells (cells/square mm)	979.94	621.05
		Percentage of positive cells	13.30	9.85

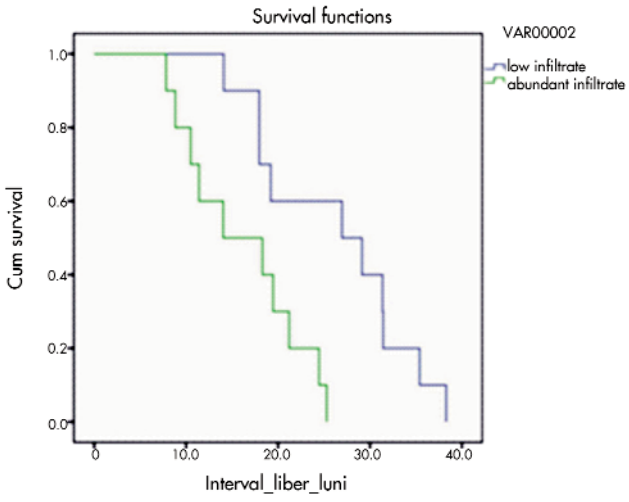

Figure 4. *Kaplan-Meier survival curve for the percentage of cd8+ T cells*

currently applied TNM staging system (29,30). While some of the studies have been aimed at finding new prognostic lymph node scores (5-12) and others have been looking more in depth at the molecular characteristics of the tumor (13-19), a new approach is taking into account the possible effect that inflammation and the immune system could have on the evolution of a colo-rectal carcinoma (23-28).

As shown above, in the results section, in our study we found a correlation between a small percentage of cd8+ T cells in the peritumoral normal tissue and a longer disease-free interval, at a level of statistical significance less than 0.05. Cd8+ T cells located in the tumor have been shown to be associated with a better overall prognosis (31,32). Under these circumstances our results might seem odd at a first glance, but when we take into account that in our study group the correlation was statistically significant only between cd8+ T cells in the peritumoral normal tissue and the disease-free survival we see that we are not discussing about inflammation located inside the tumor but inflammation situated beyond the invasive margin of the tumor. The Kaplan-Meier survival analysis also showed that the percentage of cd8+ T cells can be used to stratify the patients in terms of relapse-free prognosis.

Furthermore we were able to find a correlation between a low cd45ro+ cell density in the normal peritumoral tissue and a longer disease-free survival even though the majority

of the studies agree that memory T cells are a positive prognostic factor (33-36). However these studies focus on the intratumoral tissue and on the invasive margin of the tumor.

An interesting question that our study raises in this case is whether the cd8+ T cells and the memory T cells are the ones that stimulate an abnormal inflammation in the normal tissue that is close to the tumor or is it the other way around and through abnormal inflammatory signalling pathways the tumor induces abnormal inflammation in the normal peritumoral tissue. This is a question that is even more exciting when we consider the fact that most of the current studies have focused on studying the tumor and the invasive margin overlooking the possible importance of the abnormal inflammation occurring in the normal tissue surrounding the tumor.

Conclusion

We have shown that for the normal tissue around the tumor the cd8+ inflammatory infiltrates prognosticating a relapse-free outcome are in opposition to intratumoral infiltrates. Thus we can say that small percentages of cd8+ T cells could be predictive for longer disease-free intervals, but more extensive studies with larger study groups are necessary to confirm our results. Also we have to keep in mind that the majority of the other studies have only studied the intratumoral tissue and the invasive margin of the tumor, while the results in this study have only shown statistically significant correlations for infiltrates in the normal peritumoral tissue. All of these findings raise more questions than they answer, and it is obvious that we are barely scratching the surface when it comes to discussing the complex signalling pathways that lead to tumor invasiveness.

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