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Prognostic and Predictive Value of Lymphoid Microenvironment in Locally Advanced Rectal Cancer

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Abstract

Objective: Tumor immunity infiltrate, density and location in the microenvironment could be predictive value for survival in patients with rectal cancer. The aims of this study is to evaluate tumor microenvironment using immune adaptive cells as $CD3^+$ and $CD8^+$ T lymphocytes and explore relationship between clinic pathological features, therapeutic response, survival outcomes and immunoscore.

Materials and Methods: Scoring system "immunoscore" in pretreated biopsies tissues from rectal cancer. Quantification of cytotoxic and memory T cells in the Core of Tumor (CT) and in the tumor's Invasive Margin (IM)

Results: According to 5 immunoscore groups I0, I1, I2, I3 and I4, the frequency of each group was respectively 9.3%, 18.5%, 22.2%, 18.5% and 31.5%. When grouping immunoscore into 3 groups' data allow to a proportion of 27.8% for low immunoscore, 22.2% for moderate immunoscore and 50% for high immunoscore. Furthermore, in two-tailed classification (I0, I1, I2 versus I3 and I4) 50% of tumors were ranged as low immunoscore versus 50% as high immunoscore.

Conclusion: High Lymphoid infiltration in CT and IM prove that tumor microenvironment could be a predictive factor, on pretreated biopsies, for tumor sensibility after neoadjuvant treatment in rectal cancer. This reflects the importance role of high immune infiltration in prediction of complete therapeutic effect. Immunoscore method should be considered as predictive marker for overall survival.

Keywords: Immunoscore; CD3; CD8; Therapeutic response; Rectal cancer; Neoadjuvant treatment

Introduction

Tumor microenvironment is an extrinsic factor that modifies the behavior of the tumor cell [1]. The understanding of the tumoral properties leads to appreciate the influence of the peripheral and intratumoral immune component on the prognosis and the evolution of the cancer [2]. The immune system alone is able to recognize and eliminate precancerous cells that are undergoing cancer transformation [3]. The concept of "immune surveillance of cancer" is currently demonstrated and validated, and has been defined as a new vision of "immunoediting of cancer" [4,5]. In Addition, the tumor immune infiltrate consists of adaptive immune cells such as B and T-lymphocytes presenting a specific antigen receptor, and which are responsible for the immune memory function. Furthermore innate immune system cells are part of the tumor infiltration [2]. The "immunoscore" method has been designed by several authors to focus on the concept of the tumor microenvironment, as well as to explore and quantifies the immune infiltrate characters in solid tumors [2,5]. The European Hospital of Georg Pompidou (EHGP) immunomonitoring platform demonstrate an excellent concordance rate of eye and machine count correlation for CD3 and CD8 (r>0.80) [2]. This immunologic method has been validated especially in colorectal cancer [6]. "Immunoscore" system is based on the recognition of CD expressed on immune cells. CD4 "helper" T-lymphocytes as well as cytotoxic effector CD8 T cells can be found at different densities in the tumor microenvironment of tumor [7,8]. Moreover, better disease free survival and overall survival has been associated with a strong intra-tumoral infiltration of $CD3^+$, $CD8^+$ T and CD45R0 lymphocytes in solid tumors [9,10]. Furthermore, current research has focused on establishment of a new classification based on immunoscore (TNM-Immune), to better characterize the prognosis and behavior of patients more accurately than classical pathologic TNM as reported by 7th AJCC staging system [11,12].

For this purpose, the aim of our study is to determine whether the immune infiltrate of the tumor, recently evaluated with the immunoscore methodology, could be a useful predictive and prognostic marker in patients with rectal cancer, also to encourage the use of this method as a routine test for the classification of rectal cancer.

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Materials and Methods

Patients and pathological examination

In all, 54 patients diagnosed with adenocarcinoma at HASSAN II University Hospital Center of Fez were included in this study. Patients received neoadjuvant treatment followed by surgical procedure. We followed the methods of Otmani [13]. Samples collected and selected for Immunohistochemistry (IHC) were Formalin-Fixed-Paraffin-Embedded (FFPE) pre-therapeutic biopsies.

Histological slides based on a hematoxylin and eosin-stained slide were evaluated by a gastrointestinal pathologist. Histological parameters were investigated and performed according to the staging criteria of the American Joint Committee on Cancer, 7th edition (AJCC) (histological type, tumor differentiation, tumor regression grade with the Dworak Grading, post-neoadjuvant treatment TNM stage (ypTNM), lymph node status. And other clinic pathological characteristics). Data registered include demographic details, neoadjuvant treatment details, type and results of surgery, pathology reports, and cancer outcome.

The total density of cytotoxic CD3$^+$ and CD8$^+$ T lymphocytes were evaluated by immunohistochemistry and quantified by simple optical microscope analysis for 54 tumor biopsies taken before neoadjuvant treatment of patients with rectal cancer. Results were correlated with clinic pathological features, therapeutic response on surgical specimens after neoadjuvant therapy as well as disease progression (metastasis and recurrence) and patient survival.

Evaluation of CD3 and CD8 expression by immunohistochemistry

Multiple fine sections (3ym) were cut and mounted on super frost glass slides from the biopsy tissues blocks for subsequent immunostaining. Manual immunohistochemistry was performed according to the manufacturer's protocol. Slides incubated at 56°C during the night preceding the technique, were deparaffinized using toluene, rehydrated with serial alcohol and distilled water. EnVisionTM FLEX, High pH (Link), DAKO system was used in all Following steps. Unmasquage was performed by immersion slides in target retrieval solution 0.001 M (pH 9.0) at a preheated temperature of 65°C and then a target final temperature set at 95°C during 20 mn. Slides were immersed in a wash buffer for 5 mn and treated with peroxidase-blocking reagent for 3 mn to block endogenous peroxidase activity. Specific antibodies for each protein were used for membrane staining a CD3 DAKO monoclonal rabbit antibodies, clone 4B12, ready to use was performed. FLEX monoclonal mouse anti-human CD8, Clone C8/144B; Ready-to-use (Link) was used to evaluate density of CD8. The tissue section was then incubated during 1 hour at room temperature. HRP-system detection was used during 30 mn followed by 5 mn incubation with 3,3'-diaminobenzidine for color reaction. A counterstaining using Hematoxylin for 2 mn was performed. Final serial ethanol washes were used followed by a mounting of the slats with specific glue. The slides were analyzed and the staining of each marker was evaluated according to a specific manner.

CD3 and CD8 density in the Core of Tumor (CT) and in the tumour's Invasive Margin (IM)

The density of total CD3 T cells was determined by simple counting using an optical microscope. The number of CD3 T lymphocytes was calculated with respect to an area equal to 1mm^2. An observation with the Leica DM 2500 microscope using the x20 magnification, and an optic index equal to 1/16, allowed limiting a microscopic field of an area of 1.25 mm^2. The number obtained by 1.25 mm^2 was then calculated to obtain a density corresponding to a surface of 1 mm^2. The number of T lymphocytes was counted in the Core of Tumor (CT), choosing the richest tumor zone in lymphocyte cells on a stained slide. Moreover the lymphocytes density in tumour's Invasive Margin (IM) was calculated at the area of overlap between tumor and normal tissue [10]. This procedure was performed for both CD3 and CD8 T lymphocytes.

Immunoscore design

The median of each region was calculated; median cell densities of CD3 in the CT region (CD3$^+$ CT) and in the IM region (CD3$^+$ IM) as well as CD8 T cells in the CT region (CD8$^+$ CT) and in IM region (CD8$^+$ IM). The calculated median for each region was considered as threshold for classifying regions into two groups as high (1) or low (0) density as shown in Figure 1.

The next step in designing the immunological test "immunoscore" was to calculate the summation of scores of two markers of CD3 and CD8. Thus, immunoscore was classified in groups from I0 to I4 as described in Table 1. A low density of two markers CD3 and CD8 in two regions was defined as I0 and a high density of two markers CD3 and CD8 in two regions was defined as I4 (Table 1).

Furthermore, immunoscore was classified, for large investigation, in 3 groups of low immunoscore corresponding to I0 and I1, moderate immunoscore corresponding to I2, and a high immunoscore witch correspond to I3 and I4. Another stratification was used, based on a split into two groups of immunoscore; A first group of low immunoscore (I0, I1, and I2) and a second group of high immunoscore (I3, I4).

The immune infiltration of each marker of CD3 and CD8 was also analyzed separately. Thus a high density of combined tumor region of CT/IM was classed as High Density (HD), a Moderate Density (MD) of

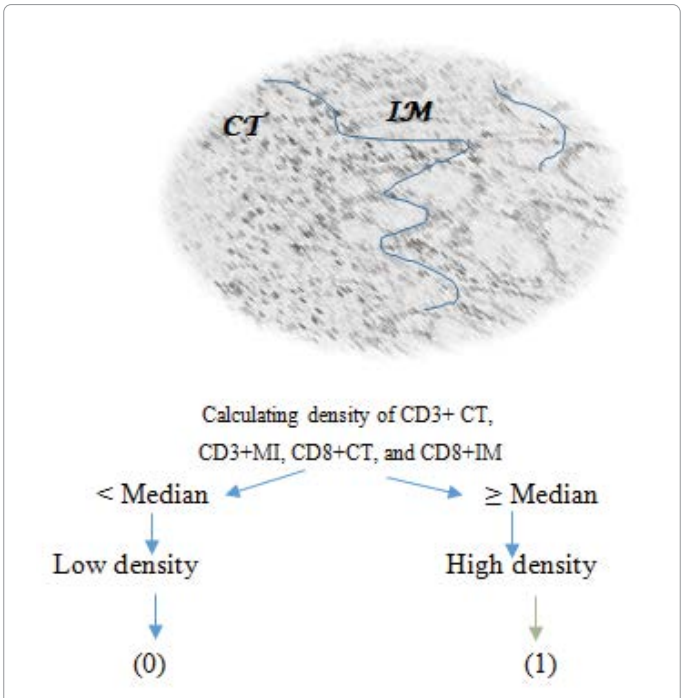

Figure 1: Chart illustrating the procedure of calculating Immunoscore. Cut-off value calculated as median density of each region of CT and IM for both CD3 and CD8.

$CD3^+$		$CD8^+$		Score
$CD3^+CT$	$CD3^+IM$	CD8+CT	$CD8^+IM$	
1	1	1	1	I4
1	1	1	0	I3
1	1	0	0	I2
1	0	0	0	I1
0	0	0	0	I0

Table 1: Immunoscore of 5 groups calculated with density summation of 2 region in 2 markers CD3 and CD8.

CD marker was defined as a summation of high and low density of two region CT and IM, and a Low Density (LD) was defined as a low density in both CT and IM region [10].

Statistical analysis

Chi-Square test and t-student test were used when appropriate to analyses association between clinic pathological features and immunoscore. Kaplan-Meier curves were used to analyses Disease Free Survival (DFS) and Overall Survival (OS). Results were considered statistically significant when $p<0.05$. The SPSS version 21 was used for statistical analysis.

Results

Clinic pathologic data

Demographic and clinopathologic features of 54 patients were investigate and shown in Table 2. The patients study comprised 22 (40.7%) men and 32 (59.3%) women with a mean age of 55.26 years (range, 28-80 years). Histologic type of 51 (94.7%) of pre-treated biopsies were diagnosed with adenocarcinoma, and 3 (5.6%) were mucinous and signet ring carcinoma. 27 (55%) tumours out of 54 were well differentiated, 24 (44%) and 3 (5.6%) were moderate and poor and undifferentiated, respectively.

In resected specimens after neoadjuvant therapy the Dvorak staging system resulting showed an incomplete therapeutic effect in 46 (85.2%) and a complete therapeutic effect in 8 (14.8%) surgical specimens. Perineural invasion and vascular invasion were observed in 6 (11.1%) and 4 (7.4%) tumours, respectively. The median follow-up duration for Overall Survival (OS) was 34 months (range, 4-80 months). Out of 54 patients, 5 (9.3%) died of tumor or other causes. The rate of relapse free survival (RFS) was 31.5% (n=17).

Immune density of CD3 and CD8

Tumor infiltration lymphocytes was analyzed using quantification of two markers of CD3 and CD8 in both CT and IM region, results were described in shows images of immunohistochemistry staining result in pre-treated rectal biopsies (Table 2 and Figure 2).

According to 5 immunoscore groups, I0, I1, I2, I3 and I4, frequency of each group was 9.3%, 18.5%, 22.2%. 18.5% and 31.5% respectively. When grouping immunoscore into 3 groups' data allow to a proportion of 27.8% for low immunoscore, 22.2% for moderate immunoscore and 50% for high immunoscore. Furthermore, in two-tailed classification (I0, I1, I2 versus I3 and I4) 50% of tumours were ranged as low immunoscore *vs* 50% as high immunoscore.

Demographic characteristics	Patients N (%)
Age, mean (year)	55.26
Minimum	25
Maximum	80
Standard deviation	14.66
Age	
<50	22 (40.7)
≥ 50	32 (59.3)
Sex	
Male	22 (40.7)
Female	32 (59.3)
Histopathology, clinical characteristics of pretreated biopsy	
Histologic type	
Adenocarcinoma	51 (94.4)
Mucinous carcinoma/signet ring carcinoma	3 (5.6)
Degrees of differentiation	
Well	27 (55)
Moderate	24 (44)
Poor/undifferentiated	3 (5.6)
Tumor location	
High rectum	4 (7.4)
Median rectum	27 (50)
Low rectum	23 (42)
Immunohistochemistry of pretreated biopsies	
Density of CD3 CT, mean (n cells/mm^2)	74.37
Median (n cells/mm^2)	60
Standard deviation	39.45
Minimum	8
Maximum	180
Density of CD3 IM, mean(n cells/mm^2)	54.28
Median(n cells/mm^2)	50
Standard deviation	29.66
Minimum	10
Maximum	130
Score of CD3 (CT/IM) (one marker/two regions)	
Ld	10 (18.5)
Md	22 (40.7)
Hd	22 (40.7)
Density of CD8 CT, mean (n cells/mm^2)	41.94
Median (n cells/mm^2)	40
Standard deviation	25.39
Minimum	10
Maximum	120
Density of CD8 IM, mean (n cells/mm^2)	29.22
Median (n cells/mm^2)	20
Standard deviation	20
Minimum	5
Maximum	80
Score of CD 8 (CI/IM) (one marker/two regions)	
Ld	14 (25.9)
Md	14 (25.9)
Hd	26 (48.1)
Immunoscore groups of 5	
I0	5 (9.3)
I1	10 (18.5)
I2	12 (22.2)
I3	10 (18.5)
I4	17 (31.5)

Immunoscore groups of 3	
L	15 (27.8)
M	12 (22.2)
H	27 (50)
Immunoscore groups of 2	
L	27 (50)
H	27 (50)
Characteristics of post therapeutic resection specimens	
Therapeutic response	
<50%	19 (35.2)
≥ 50%	35 (64.8)
Therapeutic response	
Incomplete response	45 (83.3)
Complete response	9 (16.7)
Dvorak grading system	
1	7 (13)
2	19 (35.2)
3	19 (35.2)
4	9 (16.7)
Peri-neural invasion	
Presence	6 (11.1)
Absence	48 (88.9)
Vascular invasion	
Presence	4 (7.4)
Absence	50 (92.6)
ypN	
N0	36 (66.7)
N1	13 (24.1)
N2	5 (9.3)
ypT	
T0	9 (16.7)
T1	2 (3.7)
T2	20 (37)
T3	22 (40.7)
T4	1 (1.9)
Abbrevations: CT: Core of tumor; IM: Invasive margin; Ld: Low density; Md: Moderate density; Hd: High density; L: Low; M: moderate; H: high	

Table 2: Description analysis of clinicopathologica data.

Immunoscore and relationship with clinic pathological features

We analyzed immune infiltration using immunoscore grouped into 3 methods; 5 groups, 3 groups and 2 groups. The 3 methods were correlated with clinic pathological characteristics of pre-treated rectal cancer biopsies and post therapeutic resected specimens. High immunoscore (I4) was significantly associated with complete response (p=0.003) as shown in Table 3. In addition there was significant association between high immunoscore (I4) and lower ypT stage (p=0.008), as shown in Tables 3-5. Moreover, significant association was founded between 5 groups of immunoscore and lymph node status, low immune infiltration (I0, I1 I2) was associated presence of metastasis lymph node (N1)(p=0.039).

In contrast when dividing patients into 3 and 2 groups of immunoscore, as shown in no other significant association was observed between clinicopathological parameters and Immune infiltration (Tables 4 and 5).

Considering analysis according to one marker of CD3 and CD8. Density evaluated in combined CT and IM tumor region associated with clinicopathological features was illustrated in Table 6.

Significant association was found between density of CD3 and sex, 43.8% of women had a high density infiltration compared to only 36.4 of men with a proportion of 36.4% in high score infiltration. Both CD3 and CD8 showed association with pathologic response to neoadjuvant treatment. We observed that 88.9% and 100% of CD3 and CD8, respectively, of patients who expressed high density had a complete response (p=0.008, p=0.002). Moreover, 64.5% of patients who had a T0, T1 and T3 stage was classed in High score of density infiltration of CD8 (p=0.016).

Survival analysis correlated with immunoscore

The correlation between survival analysis and immunoscore was investigated in rectal cancer. The effect of T-cell density was correlated to immune infiltration classified on 3 groups as showed in Figures 3a and 3b, a significantly association was found between OS and high expression of immunoscore (p=0.45), while RFS was not associated with immune density in different region of two markers. Patient's

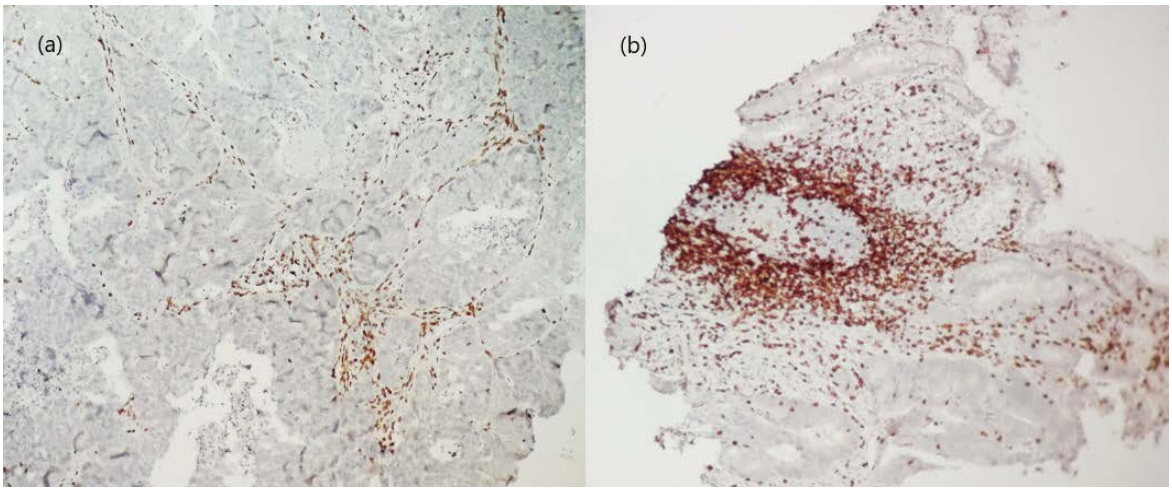

Figure 2: Immunohistochemistry showing nuclear staining. (a) Core of tumor region of CD3 (magnification x 200), (b) Invasive margin region of CD8 (magnification x200).

	Immunoscore n(%)					p value
	I0	I1	I2	I3	I4	
Gender						
Male	1 (4.5)	4 (18.2)	5 (22.7)	6 (27.3)	6 (27.3)	0.364
Female	4 (12.5)	6 (18.8)	7 (21.9)	4 (12.5)	11 (34.4)	
Age						
<50	3 (13.6)	3 (13.6)	7 (31.9)	3 (13.6)	6 (27.3)	0.516
≥ 50	2 (6.3)	7 (21.9)	5 (15.6)	7 (21.9)	11 (34.4)	
Histologic type						
Adenocarcinoma	5 (9.8)	8 (15.7)	12 (23.5)	10 (19.6)	16 (31.4)	0.239
Mucinous carcinoma/signet ring carcinoma	0 (0)	2 (66.7)	0 (0)	0 (0)	1 (33.3)	
Degrees of differentiation						
Well	2 (7.4)	7 (25.9)	5 (18.5)	6 (22.2)	7 (25.9)	0.595
Moderate	3(12.5)	2 (8.3)	7 (29.2)	4 (16.7)	8 (33.3)	
Poor/undifferentiated	0 (0)	1 (33.3)	0 (0)	0 (0)	2 (66.7)	
Therapeutic response						
<50%	3 (15.8)	4 (21.1)	3 (15.8)	6 (31.6)	3 (15.8)	0.14
≥ 50%	2 (5.7)	6 (17.1)	9 (25.7)	4 (11.4)	14 (40)	
Therapeutic response						
Incomplete response	5 (11.1)	10 (22.2)	12 (26.7)	9 (20)	9 (20)	0.003
Complete response	0 (0)	0 (0)	0 (0)	1 (11.1)	8 (88.9)	
Dvorak						
1	1 (14.3)	1 (14.3)	1 (14.3)	2 (28.6)	2 (28.6)	0.016
2	3 (15.8)	5 (26.3)	3 (15.8)	5 (26.3)	3 (15.8)	
3	1 (5.3)	4 (21.1)	8 (42.1)	2 (10.5)	4 (21.1)	
4	0 (0)	0 (0)	0 (0)	1 (11.1)	8 (88.9)	
Perineural invasion						
Yes	2 (33.3)	0 (0)	0 (0)	1 (16.7)	3 (50)	0.086
No	3(6.3)	10(20.8)	12(25)	9(18.8)	14(29.2)	
Vascular invasion						
Yes	1 (25)	1 (25)	0 (0)	1 (25)	1 (25)	0.725
No	4 (8)	9 (18)	12 (24)	9 (18)	16 (32)	
ypT						
T0	0 (0)	0 (0)	0(0)	1 (11.1)	8 (88.9)	0.008
T1	0 (0)	0 (0)	1 (50)	0 (0)	1 (50)	
T2	2 (10)	2 (10)	6 (30)	7 (35)	3 (15)	
T3	3 (13.6)	8 (36.4)	4 (18.2)	2 (4.1)	5 (22.7)	
T4	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	
YpT						
ypT0/T1/T2	2 (6.5)	2 (6.5)	7 (22.6)	8 (25.8)	12 (38.7)	0.043
ypT3/T4	3 (13)	8 (34.8)	5 (21.7)	2 (8.7)	5 (21.7)	
ypN						
N0	2 (5.6)	5 (13.9)	10 (27.8)	7 (19.4)	12 (33.3)	0.039
N1	3 (23.1)	3 (23.1)	2 (15.4)	0 (0)	5 (38.5)	
N2	0 (0)	2 (40)	0 (0)	3 (60)	0 (0)	
ypN						
N0	2 (6.5)	2 (6.5)	7 (22.6)	8 (25.8)	12 (38.7)	0.371
N1/N2	3 (13)	8 (34.8)	5 (21.7)	2 (8.7)	5 (21.7)	
Abbrevation: I:Immunoscore						

Table 3 : Association between 5 groups of immunoscore and clinicopathological features.

	Group of Immunoscore			p value
	Low	Moderate	Height	
Gender				
Male	5 (22.7)	5 (22.7)	12 (54.5)	0.77
Female	10 (31.3)	7 (21.9)	15 (46.9)	
Age				
<50	6 (27.3)	7 (31.8)	9 (40.9)	0.341
≥ 50	9 (28.1)	5 (15.6)	18 (56.3)	
Histologic type				
Adenocarcinoma	13 (25.5)	12 (23.5)	26 (51)	0.422
Mucinous carcinoma/signet ring carcinoma	2 (66.7)	0 (0)	1 (0)	
Degrees of differentiation				
Well	9 (33.3)	5 (18.5)	13 (48.1)	0.712
Moderate	5 (20.8)	7 (29.2)	12 (50)	
poor/un differentiated	1 (33.3)	0 (0)	2 (66.7)	
Therapeutic response				
<50%	7 (36.8)	3 (15.8)	9 (47.4)	0.526
≥ 50 %	8 (22.9)	9 (25.7)	18 (51.4)	
Therapeutic response				
incomplete response	15 (33.3)	12 (26.7)	18 (40)	0.005
complete response	0 (0)	0 (0)	9 (100)	
Dvorak				
1	2 (28.6)	1 (14.3)	4 (57.1)	0.012
2	8 (42.1)	3 (15.8)	8 (42.1)	
3	5 (26.3)	8 (42.1)	6 (31.6)	
4	0 (0)	0 (0)	9 (100)	
Perineural invasion				
Presence	2 (33.3)	0 (0)	4 (66.7)	0.454
Absence	13 (27.1)	12 (25)	23 (47.9)	
Vascular invasion				
Presence	2(50)	0 (0)	2 (50)	0.554
Absence	13(26)	12 (24)	25 (50)	
ypT				
T0	0 (0)	0 (0)	9 (100)	0.004
T1	0 (0)	1 (1)	1 (50)	
T2	4 (20)	6 (30)	10 (50)	
T3	11 (50)	4 (18.2)	7 (31.8)	
T4	0 (0)	1(100)	0 (0)	
ypT				
ypT0/T1/T2	4 (12.9)	7 (22.6)	20 (64.5)	0.012
ypT3/T4	11 (47.8)	5 (21.7)	7 (30.4)	
YpN				
N0	7 (19.4)	10 (27.8)	19 (52.8)	0.292
N1	6 (46.2)	2 (15.4)	5 (38.5)	
N2	2 (40)	0 (0)	3 (60)	
ypN				
N0	7 (19.4)	10 (27.8)	19 (52.8)	0.12
N1/N2	8 (44.4)	2 (11.1)	8 (44.4)	

Table 4: Association between 3 group of immunoscore and clinicopathological features.

	Immunoscore of 2 group		
	Low	High	p value
	I0/I1/I2	I3/I4	
Sex			
Male	10 (45.5)	12 (54.5)	0.782
Female	17(53.1)	15(46.9)	
Age			
<50	13 (59.1)	9 (40.9)	0.406
≥ 50	14 (43.8)	18 (56.3)	
Histologic type			
Adenocarcinoma	25 (49)	26 (51)	1
Mucinous carcinoma/signet ring carcinoma	2 (66.7)	1 (33.3)	
Degrees of differentiation			
Well	14 (51.9)	13 (48.1)	1
Moderate	12(50)	12 (66.7)	
Poor/undifferentiated			
Therapeutic response			
<50%	10 (52.6)	9 (47.4)	1
≥ 50%	17 (48.6)	18 (51.4)	
Therapeutic response			
Incomplete response	27 (60)	18 (40)	0.002
Complete response	0 (0)	9 (100)	
Dvorak			
1	3 (42.9)	4 (57.1)	
2	11 (57.9)	8 (42.1)	0.005
3	13 (68.4)	6 (31.6)	
4	0 (0)	9 (100)	
Perineural invasion			
Presence	2 (33.3)	4 (66.7)	0.669
Absence	25 (52.1)	23 (47.9)	
Vascular invasion			
Presence	2 (50)	2 (50)	1
Absence	25 (50)	2 (50)	
ypT			
T0	0 (0)	9 (100)	
T1	1 (50)	1 (50)	0.004
T2	10 (50)	10 (50)	
T3	15 (68.2)	7 (31.8)	
T4	1 (100)	0 (0)	
ypN			
ypT0/T1/T2	11 (35.5)	20 (64.5)	0.027
ypT3/T4	16 (69.6)	7 (30.4)	
ypN			
N0	17 (47.2)	19 (52.8)	0.688
N1	8 (61.5)	5 (38.5)	
N2	2 (40)	3 (60)	
ypN			
N0	17 (47.2)	19 (52.8)	0.773
N1/N2	10 (55.6)	8 (44.4)	

Table 5: Association between Two groups of Immunscore and clinicopathologic features.

	CD3 CT/IM Immunoscore			p value	CD8 CT/IM Immunoscore			p value
	I0	I1	I2		I0	I1	I2	
	N(%)	N(%)	N(%)		N(%)	N(%)	N(%)	
Gender								
Male	1 (4.5)	13 (59.1)	8 (36.4)	0.031	5 (22.7)	7 (31.8)	10 (45.5)	0.077
Female	9 (28.1)	9 (28.1)	14 (43.8)		9 (28.1)	7 (21.9)	16 (50)	
Age								
<50	4 (18.2)	9 (40.9)	9 (40.9)	1	8 (36.4)	5 (22.7)	9 (40.9)	0.375
≥ 50	6 (18.8)	13 (40.6)	13 (40.6)		6 (18.8)	9 (28.1)	17 (53.1)	
Histologic type								
Adenocarcinoma	9 (17.6)	21 (41.2)	21 (41.2)	1	13 (25.5)	13 (25.5)	25 (49)	1
Mucinous carcinoma/signet ring carcinoma	1 (33.3)	1 (33.3)	1 (1)		1 (33.3)	1 (33.3)	1 (33.3)	
Degrees of differentiation								
Well	5 (18.5)	12 (44.4)	10 (37)	0.898	6 (22.2)	11 (40.7)	10 (37)	0.147
Moderate	5 (20.8)	9 (37.5)	10 (41.7)		7 (29.2)	3 (12.5)	14 (58.3)	
poor/undifferentiated	0 (0)	1 (33.3)	2 (66.7)		1 (33.3)	0 (0)	2 (66.7)	
Therapeutic response								
<50%	3 (15.8)	10 (52.6)	6 (31.6)	0.429	8 (42.1)	4 (21.1)	7 (36.8)	0.175
≥ 50%	7 (20)	12 (34.3)	16 (45.7)		6 (17.1)	10 (28.6)	19 (54.3)	
Therapeutic response								
incomplete response	10 (22.2)	21 (46.7)	14 (31.1)	0.008	14 (31.1)	14 (31.1)	17 (37.8)	0.002
complete response	0 (0)	1 (11.1)	8 (88.9)		0 (0)	0 (0)	9 (100)	
Dvorak								
1	1 (14.3)	3 (42.9)	3 (42.9)	0.078	2 (28.6)	2 (28.6)	3 (42.9)	0.007
2	5 (26.3)	9 (47.4)	5 (26.3)		8 (42.1)	3 (15.8)	8 (42.1)	
3	4 (21.1)	9 (47.4)	6 (31.6)		4 (21.1)	9 (47.4)	6 (31.6)	
4	0 (0)	1 (11.1)	8 (88.9)		0 (0)	0 (0)	9 (100)	
Perineural invasion								
Yes	2 (33.3)	1 (16.7)	3 (50)	0.54	1 (33.3)	0 (0)	4 (66.7)	0.398
No	8 (16.7)	21 (43.8)	19 (39.6)		12 (25)	14 (29.2)	22 (45.8)	
Vascular invasion								
Yes	1 (25)	2 (50)	1 (25)	1	2 (50)	0 (0)	2 (50)	0.359
No	9 (18)	20 (40)	21 (42)		12 (24)	14 (28)	24 (48)	
ypT stage								
T0	0(0)	1 (11.1)	8 (88.9)	0.09	0(0)	0 (0)	9 (100)	0.011
T1	0 (0)	1 (50)	1 (50)		0 (0)	1 (50)	1(50)	
T2	4 (20)	10(50)	6 (30)		5 (25)	5 (25)	10 (50)	
T3	6 (27.3)	9 (40.9)	7 (31.8)		9 (40.9)	7 (31.8)	6 (27.3)	
T4	0 (0)	1 (100)	0 (0)		0(0)	1(100)	0(0)	
ypT stage								
T0/T1/T2	4 (12.9)	12 (38.7)	15 (48.4)	0.348	5 (16.1)	6 (19.4)	20 (64.5)	0.016
T3/T3	6 (26.1)	10 (43.5)	7 (30.4)		9 (39.1)	8 (34.8)	6 (26.1)	
ypN status								
N0	5 (13.9)	14 (38.9)	17 (47.2)	0.185	8 (22.2)	10 (27.8)	18 (50)	0.842
N1	4 (30.8)	4 (30.8)	5 (38.5)		5 (38.5)	3 (23.1)	5 (38.5)	
N2	1 (20)	4(80)	0 (0)		1 (20)	1 (20)	3 (60)	
ypN status								
N0	5 (13.9)	14 (38.9)	17 (47.2)	0.357	8 (22.2)	10 (27.8)	18 (50)	0.751
N1/N2	5 (27.8)	8 (44.4)	5 (27.8)		6 (33.3)	4 (22.2)	8 (44.4)	

Table 6: Association between CD 3 and CD 8 immunoscore and clinicopathological features.

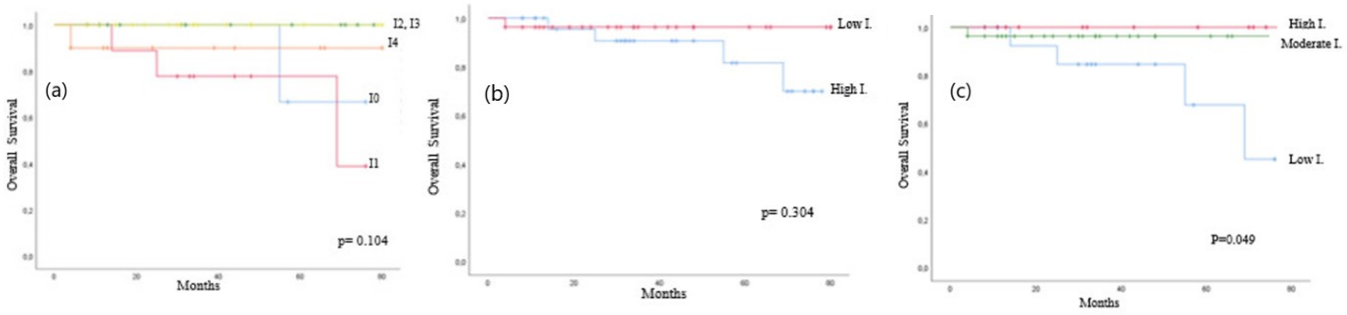

Figure 3: Overall survival curves according to 3 immunoscore classes. (a) OS according to 5 immunoscore groups. (b) OS according to 2 immunoscore groups. (c) OS according to 3 immunoscore groups.

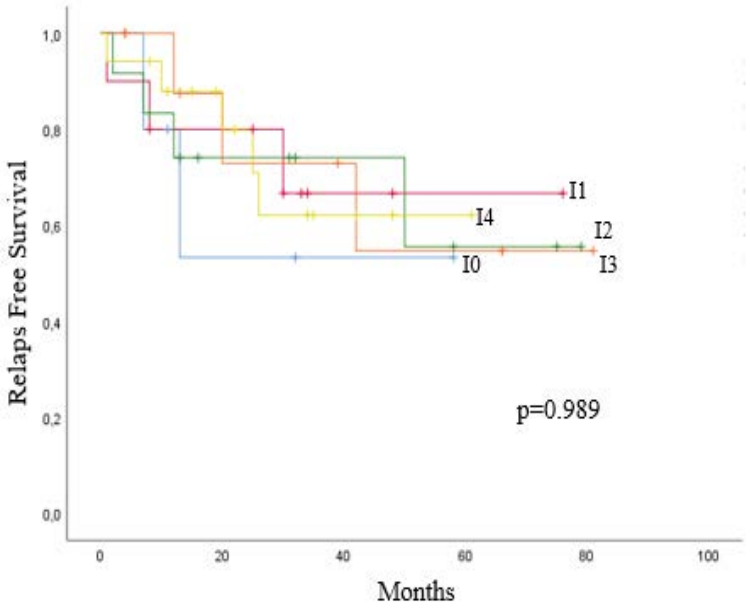

Figure 4: Kaplan-Meier curves for the time in months of RFS according to 5 Immunoscore groups.

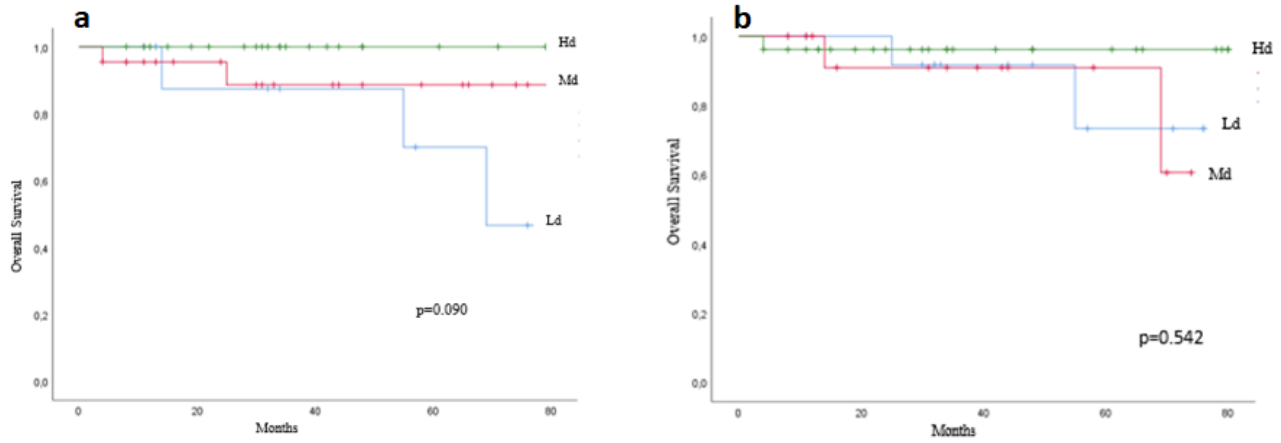

Figure 5: Kaplan-Meier curves for the time in months of OS according to density of CD3. (a) OS depending on the density of CD3 in CT and IM. (b) OS depending on the density CD3 in CT and IM. (Ld: Low density, Md: Moderate density, Hd: High density).

survival curves according to density of each marker of CD3 and CD8 did not show significance as describe in Figures 3-5.

Discussion

The interaction between immune system cells and cancer cells is part of the mechanisms of "immunosurveillance" [14]. In a global way, a new type of biomarker can define the intratumoral component in immune cells. This biomarker may be complementary to other biomarkers linked directly to the tumor cell [3]. The technological revolution, which concerned nanotechnologies, the calculation and imaging system as well as digital pathology, made it possible to better characterize and identify the component of the tumor cell with great precision and robustness. This has created a favourable ground for research and development of new biomarkers [15]. Also allowed to understand the role that the immune system plays in the dynamics of cancer and to develop an original method called "Immunoscore" [5,6]. Studies have shown that in most solid tumours such as colorectal cancer, a strong immune infiltration in the tumor has been associated with prolonged survival [9].

Our study was conducted to evaluate relationships between immune infiltration using Immunoscore test, and therapeutic response, clinicopathological features and survival outcomes of patients. We here in demonstrate that high score of immune cells infiltration in pre-treated tumours is associated with a complete pathologic response after neoadjuvant therapy. As a result, we demonstrate the importance role of densities of CD3 and CD8 in both CT and IM in therapy sensitivity.

The high level expression of immune cells of CD3 and CD8 proved necessary to ensure a complete response. This result is in accordance with publication of several studies [10,16] in which they report a significant correlation between high densities of CD3 cells and response to chemo radiation therapy evaluated using ypTNM and Dvorak classification. Similar finding was reported by Melanie J in a cohort involving 106 rectal tumours [17]. Therefore, these experiments suggested that the behavior of the tumor cell is the consequence of a balance between the tumor invasion process and the local immune response of the host [9,18]. Furthermore, this correlation can be explaining by an additional immune adjuvant acting involving innate and adaptive immune response [19]. Also previous studies on rectal cancer showed the impact of the association of cytotoxic T lymphocytes with an immune orientation of Th1 lymphocytes in tumor microenvironment [18,20,21]. Patients with a good response to neoadjuvant treatment present in the tumor area an increase in the transcript level of T lymphocyte activation genes and cytotoxic Th1 orientation [22].

We further demonstrate in our study that a high density of CD3 infiltration in both CT and IM within pre-treated rectal biopsies was correlated with a lower pathologic tumor infiltration (ypT) in rectal specimen resections after neoadjuvant treatment. The immune infiltration in tumor sites is not hindered to physical born, thus a broad expansion is confronted with an immunosuppressive environment [6,23,24]. Maria-Gabriele Anitei confirmed that the highest immunoscore I4 showed in 90% of patients was observed in localized tumor infiltration [10].

Similar research interesting 109 patients with rectal cancer was conducted by siyu Zhang between 2002 and 2005. He indicated that a highly expression of $CD4^+$ and $CD8^+$ Tumor Infiltration Lymphocytes (TIL) was correlated with more response to neoadjuvant therapy. Also he found a significant difference between TIL density between pre-therapeutic and post-therapeutic tumours [24]. This explain the major role of neoadjuvant treatment as an enhancer of immune defences by stimulating the expression of receptors specific to cell death, as well as to increase immune cell density such as $CD4^+$ and CD8, and this lead to a positive activation of tumor immune microenvironment [25].

We also highlight the performance of immune infiltration measured in pre-treated biopsies to predict the survival outcome of patients after neoadjuvant and surgical treatment [10]. Tumor microenvironment will have a major role in the development of supplementary immunotherapy cancer therapy for altered and cold tumours with combination of radiotherapy or chemotherapy with immunotherapy [6,25].

The Immunoscore will have an interest in the immune evaluation of CD3 and CD8 in diagnostic biopsies in rectal cancer in order to help in the therapeutic management. Particularly in the decision making of a minimal conservative surgery or simple monitoring [26], taking into consideration the morbidity and mortality related to the surgical procedure, as well as the alteration of the quality of life of the patients [2]. To improve significant correlation with RFS, and to obtain a comparative surgical and outcomes with literatures the respect of 10-years follow-up is required for clinical studies in rectal cancer as recommended [27].

Conclusion

Our investigational analysis highlight the role of high Immunoscore of CD3 and CD8 in both CT and IM, to be a predictive factor for pathologic complete response after neoadjuvant therapy in rectal cancer. Immune infiltration analysis of the microenvironment was correlated to improved overall survival. DFS were not found to be significant depending on microenvironment immunity infiltration. Evaluation of the immune marker on a multicentre study should be taken into consideration.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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