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ORIGINAL RESEARCH

An Immunoscore System Based On CD3$^+$ And CD8$^+$ Infiltrating Lymphocytes Densities To Predict The Outcome Of Patients With Colorectal Adenocarcinoma

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Purpose: The aim of this study was to evaluate the Immunoscore (IS) methodology as a prognostic marker of colorectal adenocarcinoma in Tunisian population. Tumor blocks were retrospectively collected from 106 patients with sporadic colorectal cancer.

Methods: Immunohistochemical staining and images analysis software were used to quantify the density of CD3$^+$ and CD8$^+$ tumor-infiltrating lymphocytes in the center of the tumor and invasive margin.

Results: The density of CD3$^+$ and CD8$^+$ was significantly associated with 5-year overall survival (P=0.001 and P=0.00098, respectively) and 5-year disease-free survival (P=0.0006 and P=0.0056, respectively). The earlier stage and the absence of vascular emboli showed a significant association with IS analysis. Cox multivariate regression analysis revealed that Immunoscore (from I0 to I4) was more significantly correlated with overall survival (P=0.00011) and disease-free survival (P=0.0008) than Tumor-Node-Metastasis (TNM) staging (P=0.057 and P=0.039, respectively). Patients with low IS were associated with inferior disease-free survival and overall survival, contrary to patients with high IS.

Conclusion: This is the first study which evaluated the prognostic value of IS methodology in colorectal cancer in African and Arabic population. The IS methodology carries out in this study allows to estimate the risk of relapse in patients with colorectal cancer. Overall, our results support the implementation of the consensus Immunoscore as a new component for the classification of cancer, designated TNM-Immune.

Keywords: colorectal cancer, immunoscore, AJCC/TNM-classification, tumor-infiltrating lymphocytes, digital pathology, immunotherapy

Introduction

The classification of colorectal cancer (CRC) is based on Tumor-Node-Metastasis (TNM) staging which allows the estimation of the prognosis of the resected tumors and then the choice of the appropriate treatment.[1–3] However, by this classification, prognosis assessments and treatment protocol can vary from patient to patient within the same histological tumor stage, hence its limitations.[4] Approximately, 20% of stage II CRC have a relapse after tumor resection.[5] Thus, many studies have tried to identify novel markers such as immunological biomarkers to expand the therapeutic arsenal and overcome TNM limits.[6] In past years, the role of tumor-infiltrating lymphocytes (TIL) as an anti-tumor immune response becomes evident.[7–9] Indeed, tumor microenvironment

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consists of many types of leukocytes such as macrophages, natural killer (NK), B lymphocytes, cytotoxic and memory T lymphocytes. Naito et al[10] were the first showing that $CD8^+$ cytotoxic T-cells represent a prognostic factor. These findings were also supported by the studies of Murphy, Nagtegaal and Chiba.[11–13] Recently, many studies showed the significant correlation between the densities of T-infiltrating lymphocytes and the prognosis of CRC. Moreover, a high density of $CD8^+$ T-lymphocytes is associated with an improved prognosis in colorectal cancer.[14] This correlation was also supported by the chemotherapy treatment efficiency at the metastatic site.[15] Among several immunological biomarkers, the ratio of $CD8^+/CD3^+$ T-cells density was recently proposed as being a significant prognostic marker in comparison to TNM staging.

Furthermore, the location, type and density of infiltrating cells in tumoral microenvironment could influence the evolution of CRC.[16] Since 2012, a novel classification called "Immunoscore" (IS) for colorectal cancer based on the quantification of $CD3^+$ and $CD8^+$ T-cell densities in the center of the tumor (CT) and in invasive margin (IM) has been proposed along with TNM staging.[17–19] An international consortium was initiated with the support of the Society for Immunotherapy of Cancer (SITC) to validate the consensus Immunoscore in clinical practice for CRC patients. The final report was published to demonstrate the significant and robust effect of IS to predict survival, local or distant tumor recurrence and treatment response.[20] In the light of all these findings cited, the aims of this current study were: (1) first, was to confirm the prognostic value of the Immunoscore for the patients with colorectal adenocarcinoma after radical surgery (2) second, was to compare accuracy of the standard TNM staging and the IS, (3) third, was to evaluate the performance of TIL to predict the choice of adjuvant treatment and (4) finely, was to demonstrate the feasibility and reproducibility of the IS method.

Materials And Methods

Patients

This study enrolled 106 Tunisian patients retrospectively assigned with sporadic colorectal adenocarcinoma diagnosed at the Department of Pathology, Charles Nicole University Hospital (CNUH), Tunis, Tunisia between January 2007 and December 2010. All patients have undergone a primary resection of the colon cancer tumor and a mesorectum excision for rectal cancer. Demographics information of patients (sex and age), tumor features, the American Joint Committee on Cancer (AJCC)/TNM staging system (I-IV), anatomic site, histological grade, vascular-lymphatic and perineural invasions were obtained from pathologic reports. Cases having an age ≤40 years were considered as young patients. Information is about surgery, adjuvant treatment and survival outcomes were obtained from medical records archives. Adjuvant chemotherapy (Folfox 4, Xeloda and/or, Folfiri) was administrated to 51 patients. Only one patient received an adjuvant radiotherapy. The mean period of follow-up was 52 months [0–115 months].

Pathological Study

The hematoxylin and eosin (H&E) sections were analyzed by two pathologists. Each pathologist gave information following criteria of the World Health Organization (WHO)[21] about tumor localization (distal, proximal and rectum), differentiation grade (well, moderate and poor), histological type (non-mucinous and mucinous cancers were those containing more than 50% of extracellular mucin), vascular emboli (VE) or lymphatic invasion (LI) or perineural invasion (PI) (VELIPI status), TNM staging system (7th edition) and macroscopic aspects. The lymph node ratio (LNR) is defined as the number of positive lymph nodes divided by the total number of lymph nodes examined.[22]

Immunohistochemical Staining

Different steps were taken: sections of 4 µm thickness were cut from paraffin tissue blocks and mounted on silanized slides. Antigen retrieval solution (10X concentrate, Novocastra, Leica), primary antibodies (Rabbit monoclonal recognizing human CD3 (Ventana Medical Systems Cat# 790-4341, RRID: AB_2335978) and CD8 (Ventana Medical Systems Cat# 790-4460, RRID: AB_2335985)) and secondary antibody (rabbit-anti-mouse IgG, Bond Refine Detection Kit, Leica) were performed according to the manufacturer's recommendations in an automate Bench Mark Ventana. Finally, sections were subsequently incubated with 3,3-diamino-benzidine (DAB^+ chromogen, Novolink, Leica), counterstained with Haematoxylin (Novocastra, Leica) and mounted with a special glue (Eukitt, GmbH, Medite). The internal positive control was used for quality assurance.

Quantification Of Tumor-Infiltrating Lymphocytes And Determination Of The Immunoscore

Slides were scanned with NanoZoomer scanner 2.0-HT (Hamamatsu C9600-02) and the acquired images were processed using the Architect XD software (Definiens

Developer XD 2.0). Image analysis software with dedicated Immunoscore module (Plug-in, INSERM/AP-HP, Paris, France) was used to determine the mean staining intensities of each slide, allowing a better sensitivity and avoiding underestimation of the total cell count (Figure 1A and B). A total of 412 images of the center of tumors (CT) and their invasive margin (IM) were analyzed to quantify $CD3^+$ and $CD8^+$ T-cell densities. In fact, the CT was defined as the region containing stroma and intra-tumoral cells and the IM was defined as the region of 200–500 µm between tumor microenvironment and normal mucosa, chosen by the software after manual delimitation. The best-performing algorithm to measure the IS has been described in the large international retrospective validation cohort led by the Society for Immunotherapy of Cancer (SITC).[17] For each marker ($CD3^+$ and $CD8^+$) and each region (CT and IM), a percentile is derived from these distributions and an average percentile is calculated based on these four values. Patients were stratified according to IS reported as I0, 1-2-3-4 based on the following average percentile classes, respectively: [0%; 10%] - [>10%; 25%] - [>25%; 70%] - [>70%; 95%] - [>95%; 100%].[4,18,19] Scores I0 and I1 corresponding to low-infiltrating lymphocytes densities of $CD3^+$ and $CD8^+$, I2 to moderate density, while I3 and I4 to high densities. Overall, a variability of the mean density between the patients was observed in each score (CD3: min = 10.1 - max = 6291 cells/mm^2; CD8: min = 3.2 - max =3017 cells/mm^2).

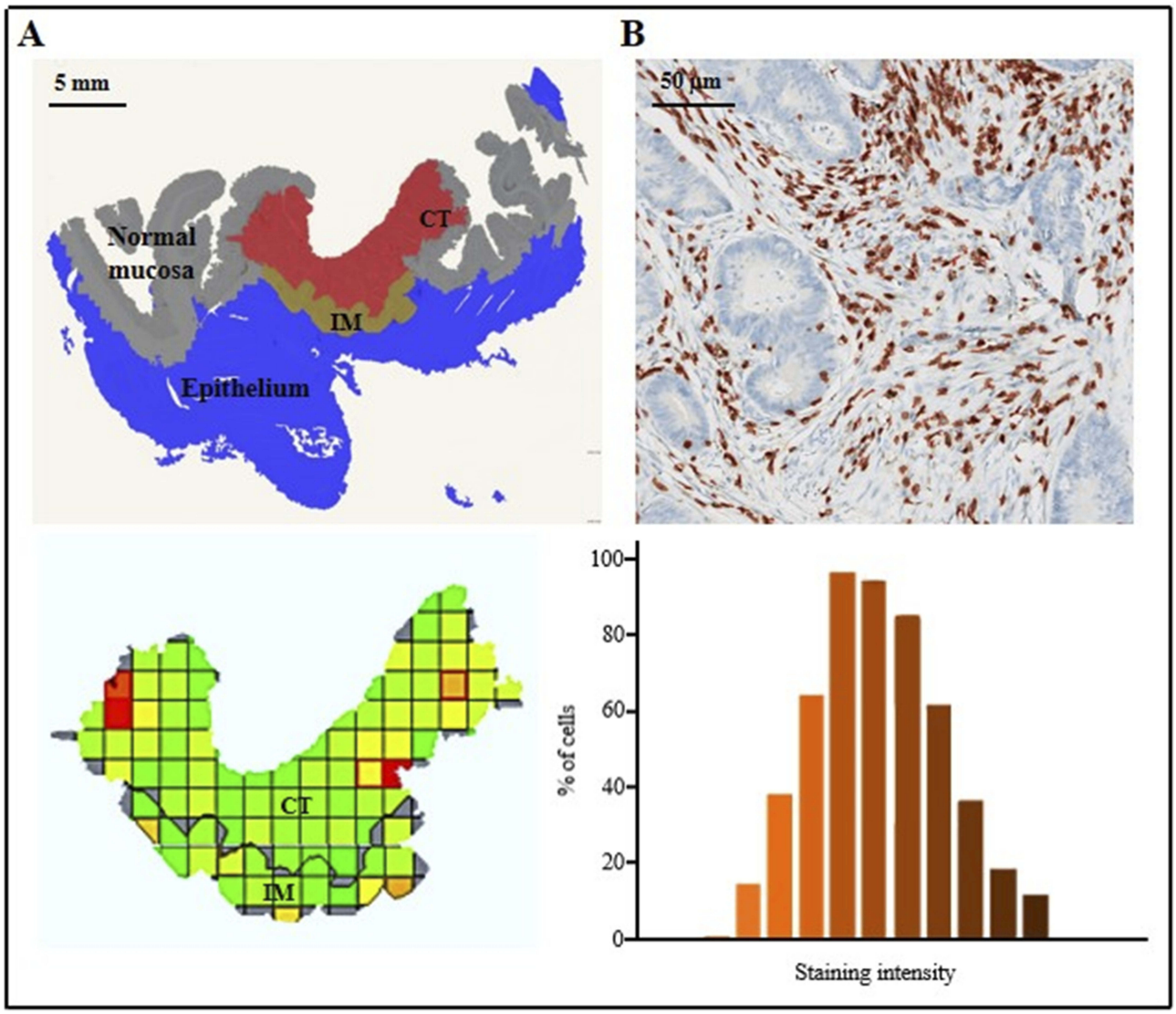

Figure 1 Image analysis software (with Immunoscore module) used to determine the infiltration T-cell densities. (**A**) The colorectal tissue is divided into tiles including center of tumor (CT) and invasive margin (IM). (**B**) Immunohistochemistry of colorectal tumor stained for $CD3^+$ T-cells (Top, in brown), and histogram of the staining intensities of positive cells detected by software leading to a valid counting (Bottom: mean brown intensity ~242 arbitrary units; middle bar chart).

Statistical Analysis

A statistical study was performed using Statistical Package for the Social Sciences (SPSS, version 19.0) and R Software (survival package, version 3.3.0). The survival data were analyzed by establishing survival curves according to the Kaplan-Meier Method (Log Rank test). Survival was divided into overall survival (OS: period between the first and last examination) and death-free survival (DFS: period between first examination and relapse). To identify the prognostic survival factors, we used an univariate analysis method (factor by factor) and a multivariate Cox regression analysis was performed for identifying the risk factors independently associated to survival (OS and DFS).

Results

Clinical And Pathological Data

Overall, 106 patients with colorectal cancer were included (Table 1). The sex ratio (Men/Women: 64/42) was equal to 1.5 and the mean age for Men was 62.07 years [33 to 84 years], whereas for Women it was 61.98 years [25 to 88 years]. 11.32% of our cases were young patients (≤40 years). We have noted the accidental discovery of the disease in 17 cases (16%). The distal colon was the predominant tumor location in 55.66% of cases and the most histological type was non-mucinous adenocarcinoma (90.6%). The majority of the tumor was well differentiated (67.96%). The features of poor prognostic, node metastasis, visceral metastasis and VELIPI were present in 44.3% of cases. Twenty-six of total metastatic patients (n=39) had metastasis in the time of diagnosis, mainly in the liver. Fifty-one patients have received an adjuvant treatment including 7 patients stage II with a high risk of relapse (presence of VELIPI criteria), 15 patients stage III and 29 patients stage IV. Different protocols were administrated for secondary localization including orally with Xeloda (300 mg/m^2/day) for two weeks and intravenously with Folfox (Eloxatin, 5-Fluorouracil and Folinic acid or Oxaliplatin, 5-Fluorouracil and folinic acid) for six cycles or Folfiri (Compto, 5-Fluorouracil and Folinic acid) for three cycles. Protocols, number of cycles and doses vary according to the anatomopathological status of the patient. Univariate analysis showed that OS and DFS are influenced by T stage, N stage, TNM staging, LNR, VELIPI criteria, and $CD3^+_{CT/IM}$ and $CD8^+_{CT/IM}$ infiltrating lymphocytes (Table 2).

Table 1 Demographic And Clinicopathological Features Of 106 Primary CRCs Patients

Parameters	No. (%)
Age (years)	
<65	43 (40.6)
65–75	42 (39.6)
>75	21 (19.8)
Tumor type	
ANM	96 (90.6)
AM	10 (9.4)
LNR	
0	59 (55.6)
<0.33	35 (33.1)
0.33–0.66	8 (7.5)
>0.66	4 (3.8)
T stage	
pTis-1	3 (2.8)
pT2	16 (15.1)
pT3	67 (63.2)
pT4	20 (18.9)
N stage	
N-	55 (51.9)
N+	51 (48.1)
TNM scoring	
I	16 (15.1)
II	31 (29.2)
III	20 (18.9)
IV	39 (36.8)
VELIPI	
Presence	59 (55.7)
Absence	47 (44.3)
CD3 (CT/IM) Score[a]	
Lo-Lo	13 (13.5)
Het	33 (34.4)
Hi-Hi	50 (52.1)
CD8 (CT/IM) Score[b]	
Lo-Lo	26 (27.37)
Het	49 (51.58)
Hi-Hi	20 (21.05)
Immunoscore[c]	
≤2	24 (26)
>2	68 (74)
Associated polyps	
Presence	34 (32.1)
Absence	72 (67.9)

Notes: [a]NA for 10 patients. [b]NA for 11 patients. [C]NA for 14 patients.

Table 2 Univariate Analysis For Overall Survival (OS) And Disease-Free Survival (DFS) Among Patients With Colorectal Adenocarcinoma

Parameters	OS			DFS		
	5-Years % (95% CI)	HR (95% CI)	*P Value*	5-Years % (95% CI)	HR (95% CI)	*P Value*
Age (years)						
<65	59.9 (41.6–86.1)	1.0 (reference)		77.4 (60.1–99.6)	1.0 (reference)	
65–75	59.6 (46.5–76.5)	1.03 (0.67–1.56)	0.904	71.3 (57.6–88.3)	1.12 (0.62–2.0)	0.708
>75	51.3 (38–69.3)	1.43 (0.75–2.74)	0.272	67.8 (53.1–86.6)	1.21 (0.50–2.91)	0.668
Tumor type						
ANM	57.1 (47.9–68.2)	1.0 (reference)		72.2 (62.2–83.1)	1.0 (reference)	
AM	50 (26.9–92.9)	1.27 (0.50–3.21)	0.618	66.7 (41.5–100)	1.43 (0.43–4.79)	0.225
LNR						
0	82.8 (73.2–93.7)	1.0 (reference)		88.0 (79.5–97.5)	1.0 (reference)	
<0.33	25.0 (7.5–83.0)	2.78 (1.65–4.7)	<0.0001	47.5 (30.9–72.9)	2.37 (1.2–4.5)	0.0006
0.33–0.66	36.6 (23.1–57.9)	2.97 (2.05–4.29)	<0.0001	47.2 (18.8–71.3)	2.54 (1.25–5.2)	0.0031
>0.66	NA (NA-NA)	5.79 (2.62–12.75)	<0.0001	NA (NA-NA)	5.81 (2.8–15.5)	<0.0001
T stage						
pTis-1	100 (100–100)	1.0 (reference)		100 (100–100)	1.0 (reference)	
pT2	93.8 (82.6–100)	2.22 (1.19–4.16)	0.010	80.4 (62.7–100)	1.33 (0.49–3.70)	0.576
pT3	54.9 (43.9–68.7)	4.35 (1.59–12.5)	<0.0001	69.7 (58.2–96.0)	1.49 (0.72–3.12)	0.262
pT4	25 (11.7–53.4)	NA (NA-NA)	0.040	60.8 (38.5–83.4)	NA (NA-NA)	0.235
N stage						
N-	81.3 (71.5–92.5)	1.0 (reference)		88.0 (79.5–97.5)	1.0 (reference)	
N+	28.5 (18.1–45.0)	6.34 (3.11–12.9)	<0.0001	44.6 (29.9–66.5)	2.90 (1.47–5.72)	<0.0001
TNM scoring						
I	100 (100–100)	1.0 (reference)		96.7 (90.5–100)	1.0 (reference)	
II	86.5 (75.0–99.7)	3.33 (1.47–7.69)	0.002	93.8 (82.6–100)	2.90 (1.47–5.72)	0.0009
III	64.6 (46.6–89.6)	4.0 (2.44–7.14)	<0.0001	77.8 (82.6–100)	6.67 (2.44–20)	<0.0001
IV	9.6 (3.4–27.1)	NA (NA-NA)	<0.0001	15.0 (4.7–48.6)	7.14 (1.89–25)	<0.0001
VELIPI						
Absence	75 (64.5–87.3)	1.0 (reference)		77.6 (97.2–89.6)	1.0 (reference)	
Presence	34 (22.5–51.3)	4.11 (2.16–7.81)	<0.0001	60.5 (45.0–81.2)	2.75 (0.92–21.1)	0.007
Associated polyps						
Absence	78.7 (65.8–94.1)	1.0 (reference)		73.2 (58.9–91.0)	1.0 (reference)	
Presence	46.2 (35.9–59.5)	1.85 (0.20–11.11)	0.0066	70.8 (59.6–84.1)	1.88 (0.99–3.58)	0.780
CD3 (CT/IM) Score[a]						
Lo-Lo	5.2 (2.7–11.4)	1.96 (1.12–3.22)	0.001	9.8 (1.7–52.4)	2.13 (1.28–3.54)	0.00006
Het	51 (38.2–68.2)	1.03 (0.66–1.59)	NA	16.4 (10.3–23)	1.23 (1.61–94.54)	0.00092
Hi-Hi	69.9 (56.6–86.1)	1.0 (reference)		71.3 (61.7–82.0)	1.0 (reference)	
CD8 (CT/IM) Score[b]						
Lo-Lo	18.7 (11.0–32.4)	1.96 (1.26–3.12)	0.0098	15.0 (7.9–28.5)	1.40 (1.30–3.12)	0.0056
Het	57.3 (46.5–70.8)	1.10 (0.65–1.87)	0.0065	25.3 (21.9–30.0)	1.14 (0.65–1.87)	0.0423
Hi-Hi	66.7 (44.7–99.5)	1.0 (reference)		60.9 (42.6–73.1)	1.0 (reference)	
Immunoscore[c]						
≤2	20.0 (10.1–30.4)	1,29 (1.04–8.33)	<0.0001	26.8 (17.2–42.5)	1.76 (0.29–4.14)	<0.0001
>2	69.7 (45.2–100)	1.0 (reference)		41.3 (28.8–51.6)	1.0 (reference)	

Notes: All *p* value ≤0.05 was considered as significant. [a]NA for 10 patients. [b]NA for 11 patients. [C]NA for 14 patients.

Abbreviations: HR, hazard ratio; CI, confidence interval; LNR, Lymph Node Ratio; NA, not assigned; TNM, tumour node metastasis; CT, centre of the tumor; IM, invasive margin. VELIPI show the presence of vascular emboli (VE) and/or lymphatic invasion (LI) and/or perineural invasion (PI); ANM, adenocarcinoma non-mucinous; AM, adenocarcinoma mucinous.

Analysis Of TIL

The cases with high density in CT and IM regions were classified as High-High "Hi-Hi" (Figure 2A). Those who are with a high density in a single region (CT or IM) for one marker were considered Heterogenous "Het" (Figure 2C) and those who are with low densities in both regions were classified as Low-Low "Lo-Lo" (Figure 2B). In our study, both densities of $CD3^+$ and $CD8^+$ T-cells were lower in tumor tissue compared with invasive margin. A significant correlation was found between $CD3^+$ and $CD8^+$ T-cells density in IM (r_0.26) (Table 3). A combined analysis for both regions (CT and IM) of the same marker ($CD3^+$ or $CD8^+$) was performed and a significant association was found between survival (OS: Figure 3 and DFS: Figure 4) and the densities of T-infiltrating lymphocytes.

Evaluation Of The IS

The scoring system depends on the total number of high densities of $CD3^+_{CT/IM}$ and $CD8^+_{CT/IM}$. 4% of our cases presented an I0 score, 10% an I1 score, 12% an I2 score, 42% an I3 score and finely 32% an I4 score. The decreasing risk of relapse was inversely proportional to IS. Kaplan-Meier analysis showed a strong association between lower IS (IS≤2: I0-I2) and shorter OS and DFS, and between higher IS (>2: I3 and I4) and longer OS and DFS (P <0.0001 for DFS and OS) (Table 2). Survival curves illustrating the overall survival and disease-free survival with the IS system are shown in Figure 5. Cox multivariate regression model (IS and TNM staging) showed that IS has a highly significant correlation with OS (HR: 2.70; P=0.0001) and DFS (HR: 2.10; P=0.0008) compared to TNM staging system (HR: 1.92; P=0.057 for OS and HR: 1.95; P=0.039 for DFS) (Table 4). This result underlines that IS can be considered the highly significant prognostic factor.

Discussion

For over 80 years, the most common system for classifying cancer, especially colorectal cancer, was the TNM staging, which gives incomplete information about prognostic and clinical outcome among patients with the same histological tumor stage.[1–3] Indeed, TNM staging does not take into account the host immune response and focuses only on the tumor cells.[23] From the beginning of the twenty-first century, growing evidence supports the important role of the immune response in the tumor. Moreover,

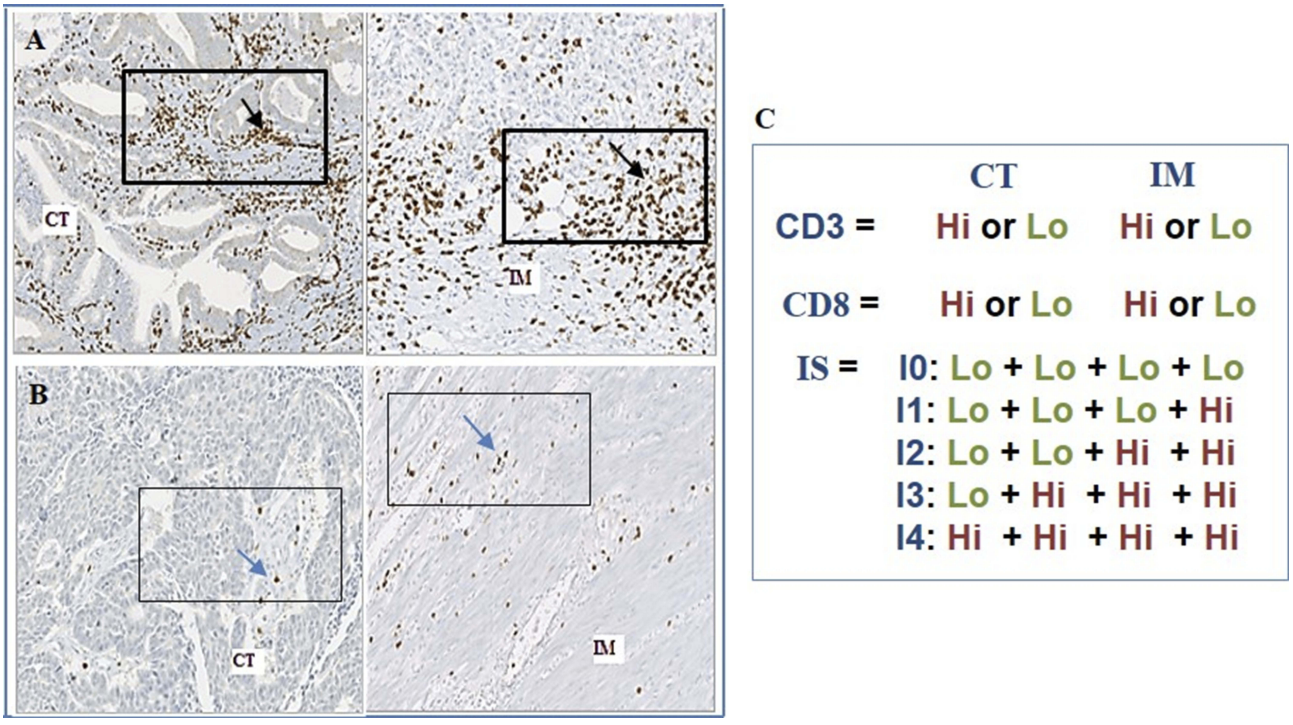

Figure 2 Representative figures of immunohistochemistry for tumor-infiltrating $CD8^+$ immune cells and schematic description of the Immunoscore model. (**A**) Immunostaining for $CD8^+$ illustrates a high number (black arrow) of positive T-cells in the CT (Left) and IM (right) regions. (**B**) Immunostaining for $CD8^+$ illustrates a low number (Blue arrow) of positive T-cells in CT (Left) and IM (Right) regions (Magnification x200). (**C**) The IS model is based on the quantification of $CD3^+$ and $CD8^+$ in the CT and IM. All patients were grouped into high-density (Hi in dark square) and low-density (Lo in light square). Score I0 correspond to low infiltrating lymphocytes densities of $CD3^+/CD8^+$ in both regions (CT plus IM), while score I4 correspond to high densities of $CD3^+/CD8^+$ in both regions.

Table 3 Association Between T-Infiltrating Lymphocytes Densities In The Center Of The Tumor And Invasive Margin Tissues

Features	Correlation Coefficient ®	*P* value
Tumor tissue		
$CD3^+_{CT}$ *vs* $CD8^+_{CT}$	0.14	0.0176
Invasive margin tissue		
$CD3^+_{IM}$ *vs* $CD8^+_{IM}$	0.26	<0.0001
Tumor *vs* invasive margin		
$CD3^+_{CT}$ *vs* $CD3^+_{IM}$ $CD8^+_{CT}$ *vs* $CD8^+_{IM}$	0.80 0.84	<0.0001 <0.0001

Note: All p value ≤0.05 was considered as significant.
Abbreviations: CT, the centre of tumor, IM, invasive margin.

the ability to avoid immune escape was introduced as another hallmark in the study of the tumor microenvironment.[24]

The determination of novel markers will allow us to choose a better-personalized treatment avoiding under/over treatment for CRC patients. Several data collected from some colorectal cancer cohort show that the presence of infiltrating lymphocytes in primitive tumors improves prognostic values for OS and DFS.[25–29] Galon and Pagès showed that tumor-infiltrating lymphocytes, especially with $CD3^+$, are directly correlated with micro-invasive status and the presence of $CD8^+$ T-cells in the center of the tumor suggests their essential role in the immune response and disease outcome.[2,30] However, the anti-tumoral immunity promotes the immunoediting, process which enables the emergence of tumor cells.[31–35] Since 2012, the new classification called "IS", based on the quantification of $CD3^+$ and $CD8^+$ T-cells densities in the CT and IM was proposed[18,36] as a strong prognostic and predictive factor and it is now endorsed by many studies.[6,37–40] $CD3^+$ and $CD8^+$ T-cells were chosen as markers, because of the quality of the staining and the stability of these antigens.

In this context, we aimed to evaluate the prognostic value of IS in the Tunisian population. Our result showed a statistically significant difference between $CD8^+_{IM}$ and $CD8^+_{CT}$ (P <0.0001), which is concordant with several studies.[19,40–42] Percentages of $CD3^+$ and $CD8^+$ T-cell densities are inversely proportional, in both CT and IM, with tumor proliferation stage (from I to IV). These results were in line with publications showing a beneficial impact of cytotoxic T lymphocytes with various tumors: colorectal, breast, melanoma, bladder, ovarian, renal, and lung.[2,29,43–45] These data suggest that tumor escape should be considered as a result of the balance between tumor infiltration mechanism and host immune response.[29,46]

In addition, our result confirms the importance of IS in the center of the tumor similarly as a prognostic and predictive novel marker. This is sustained with earlier literature reports.[6,19,41] On the other hand, the high number of $CD3^+$ and $CD8^+$ infiltrating T-cells are confirmed as being better predictive factors for survival in comparison with other immune-related cells.[2,18,47–49] In this study, CRC patients

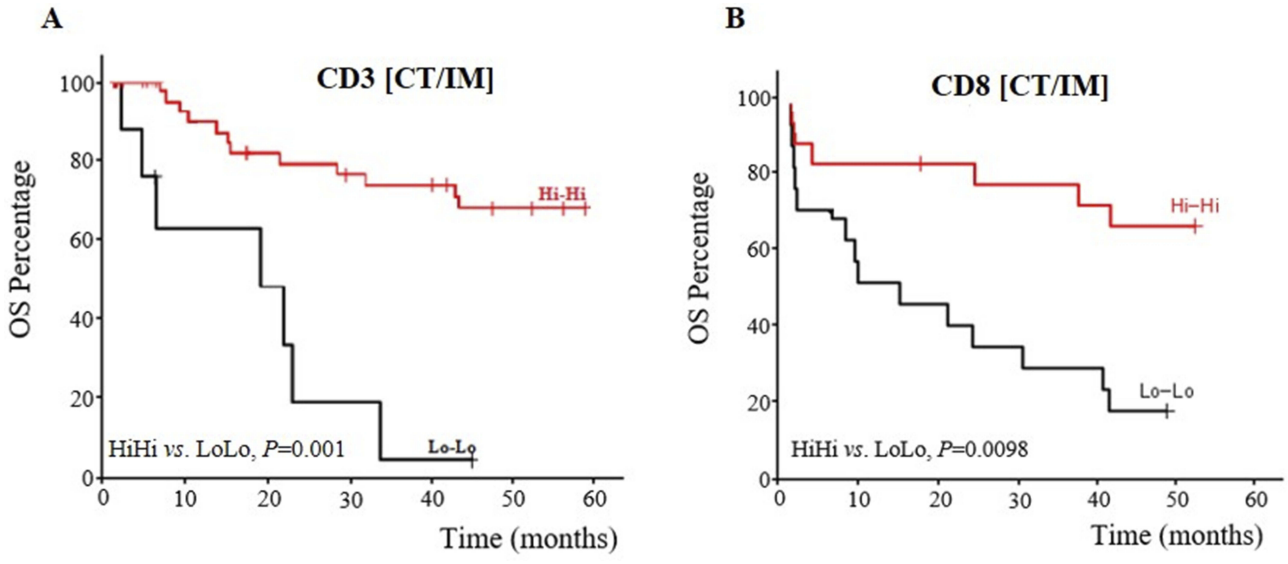

Figure 3 A Kaplan-Meier estimates of overall survival. (**A**) Kaplan-Meier curve for overall survival according to the tumor-infiltrating lymphocytes $CD3^+$ (**B**) Overall survival according to the tumor-infiltrating lymphocytes $CD8^+$. For each marker ($CD3^+$ and $CD8^+$), we observed a significant difference (P <0.005) between patients with low densities (Lo-Lo; black line), and high densities (Hi-Hi; red line).

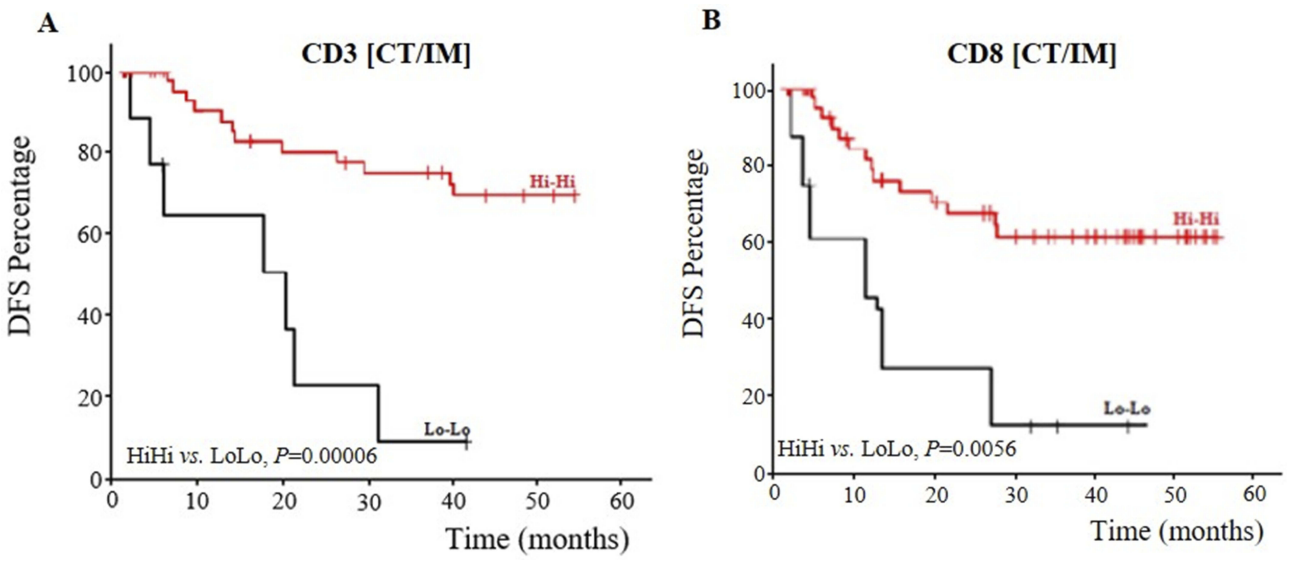

Figure 4 A Kaplan-Meier estimates of disease-free survival. (**A**) Kaplan-Meier curve for disease-free survival according to the tumor-infiltrating lymphocytes $CD3^+$. (**B**) Overall survival according to the tumor-infiltrating lymphocytes $CD8^+$.

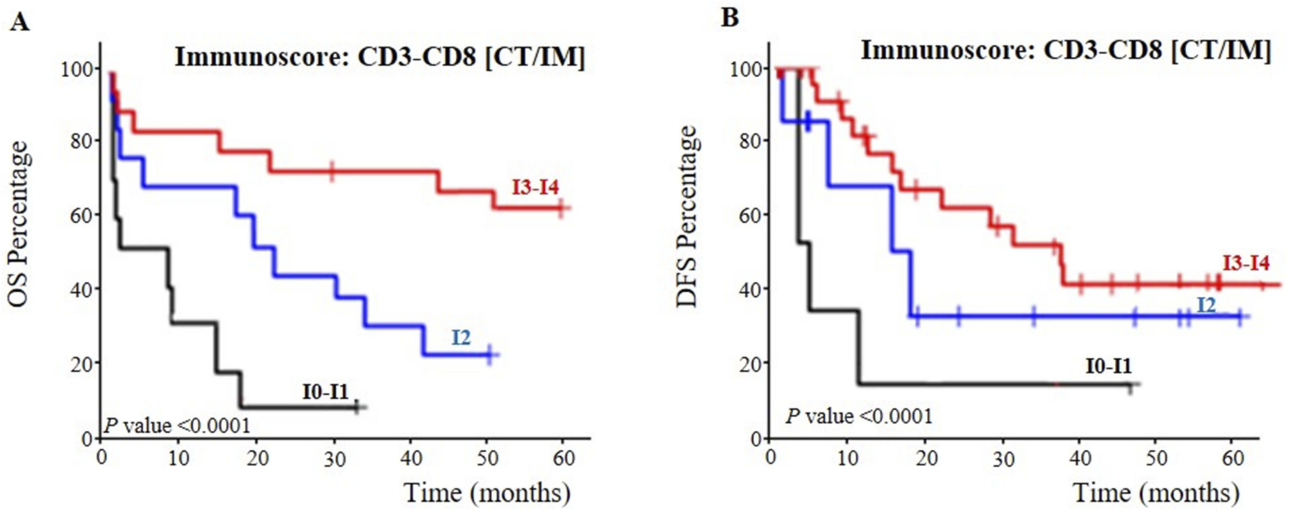

Figure 5 Kaplan-Meier estimates of survival. (**A**) Disease-free survival according to the Immunoscore of patients with colorectal adenocarcinoma. (**B**) Overall survival according to the Immunoscore. Patients with an Immunoscore ≤2 (I0, I1 and I2) experienced a poor postoperative outcome and thus could be grouped together. Patients with an Immunoscore >2 (I3 and I4) experienced a good postoperative outcome and thus could be grouped together.

having lower IS (from I0 to I2) in advanced-stage tumors (III and IV-TNM staging) showed poor outcomes compared with patients with higher IS (Figure 5A and B). The univariate analysis confirmed this result (P <0.0001 for OS and DFS) and the multivariate analysis confirms that IS is more significant than the TNM scoring system. This finding is consistent with many reports.[37,40,41] Cox model regression shows that DFS has a strongly significant correlation with IS (P=0.0008) compared to the TNM staging system (P= 0.039), which is also available for OS (Table 4). These results were in concordance with the study of Anitei et al.[19]

Pagès et al[20] were found, in their study concerning the international validation of the consensus Immunoscore for colon cancer, that the ability of IS to predict overall survival was superior to that of existing tumor risk factors such as VELIPI criteria, mucinous colloid type, differentiation, and MSI status. In fact, the predictive role of immunoprofiling will become a fundamental tool for patients' management. Typically, the tumors develop multiple mechanisms to evade the endogenous immune response, including "immune checkpoints" that can terminate immune responses after antigen activation. The immune checkpoints are necessary for

Table 4 Multivariate Cox Regression Analysis For IS And TNM Stage That Correlate With Overall Survival And Disease-Free Survival

	OS		DFS	
	HR (95% CI)	***P Value***	**HR (95% CI)**	***P Value***
Model A, before stepwise (step AIC) selection				
LNR	0.60 (0.41 to 0.98)	0.269	0.65 (0.39 to 0.95)	0.254
T Stage	2.90 (1.77 to 4.73)	<0.0001	2.04 (0.86 to 3.0)	0.0021
N Stage	0.87 (0.76 to 4.59)	0.171	0.73 (1.14 to 2.53)	0.392
VELIPI	4.02 (2.56 to 6.3)	<0.0001	4.65 (2.47 to 8.74)	<0.0001
Associated polyps	0.93 (0.42 to 1.85)	0.863	0.91 (0.39 to 2.29)	0.858
Immunoscore[a] (I0 to I4)	3.29 (1.42 to 7.15)	0.0007	3.44 (1.97 to 7.51)	0.00065
Model B, after stepwise (step AIC) selection				
T stage	3.29 (2.24 to 5.81)	<0.001	3.15 (2.20 to 4.98)	<0.001
VELIPI	5.14 (1.60 to 21.11)	<0.0001	5.63 (2.03 to 27.62)	<0.0001
Immunoscore[a] (I0 to I4)	2.59 (2.08 to 4.89)	0.007	2.03 (1.00 to 5.02)	0.0013
Model C				
TNM stage	1.92 (1.03 to 2.07)	0.057	1.95 (0.98 to 2.06)	0.039
Immunoscore[a] (I0 to I4)	2.70 (1.80 to 5.11)	0.00011	2.10 (1.13 to 4.16)	0.0008

Notes: All categorical covariates were transformed into numeric codes before Cox model analysis. Model C: Cox multivariate regression analysis by adding TNM stage to IS after stepwise selection. Correction using C= 1-(SE [coef]/coef);[2] heuristic shrinkage factor corrected with Holländer et al [a]leave-one-out method.
Abbreviations: OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; AIC, Akaike Information Criterion; LNR, Lymph Node Ratio; TNM, Tumor, node and metastases; VELIPI, vascular emboli, lymphatic invasion and perineural invasion.

developing immunotherapeutic approaches, especially for colorectal cancer.[6] Thus, the use of Immunoscore, as a novel strategy in clinical routine, is necessary to assess the prognostic and predictive values accurately and to choose the best therapeutic choice for patients.

Conclusion

To summarize, the TNM staging system is widely used to evaluate CRC prognosis, but unfortunately, it cannot predict the response of treatment. The reproducibility and robustness of the IS methodology as a strong prognostic marker favor its implementation as a new component in the classification of cancer, TNM-Immune. Moreover, the IS has a strongly significant effect for predicting survival, treatment response and local or distant tumor relapse. The combined analysis of $CD3^+$ and $CD8^+$ IS markers was not only reliable for prognosis but was also very useful to choose the best cancer treatments. Patients presenting a low rate of infiltrating T-cells will require additional treatment to chemotherapy with antibodies which allow reactivating the anti-tumoral immune response. This first investigation will serve as a working model, to apply it to a larger number of patients and also, to other African and Arab population.

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Ethics Approval And Consent To Participate

The study was approved by the Ethics Committee of Salah Azaïz Cancer Institute (ISA/2018/22) and all the samples were obtained with written informed consent and analyzed anonymously. The study was conducted in accordance with the Declaration of Helsinki.

Disclosure

The authors declare that they have no competing interests in this work.

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