Prognostic and Predictive Values of the Immunoscore in Patients with Rectal Cancer

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Abstract

Purpose: To determine whether the tumor immune infiltrate, as recently evaluated with the Immunoscore methodology, could be a useful prognostic marker in patients with rectal cancers.

Experimental design: The influence of the immune infiltrate on patient's outcome was investigated in patients with or without preoperative chemoradiation therapy (pCRT). The density of total (CD3+) and cytotoxic (CD8+) T lymphocytes was evaluated by immunohistochemistry and quantified by a dedicated image analysis software in surgical specimens of patients with rectal cancer (n = 111) who did not receive pCRT and in tumor biopsies performed before pCRT from additional 55 patients. The results were correlated with tumor recurrence, patient's survival, and response to pCRT.

Results: The densities of CD3+ and CD8+ lymphocytes and the associated Immunoscore (from I0 to I4) were significantly correlated with differences in disease-free and overall survival (HR, 1.81 and 1.72, respectively; all P<0.005). Cox multivariate analysis supports the advantage of the Immunoscore compared with the tumor–node–metastasis (TNM) staging in predicting recurrence and survival (all P<0.001). Lymph node ratio added information in a prognostic model (all P<0.05). In addition, high infiltration of CD3+ and CD8+ lymphocytes in tumor biopsies was associated with downstaging of the tumor after pCRT (CD3+ cells; Fisher exact test P = 0.01).

Conclusions: The Immunoscore could be a useful prognostic marker in patients with rectal cancer treated by primary surgery. The determination of the immune infiltrate in biopsies before treatment could be a valuable information for the prediction of response to pCRT. Clin Cancer Res; 20(7); 1891–9. ©2014 AACR.

Introduction

In rectal cancer, the worldwide used American Joint Committee on Cancer/Union Internationale Contra Cancer tumor–node–metastasis (AJCC/UICC-TNM) system (TNM; ref. 1) is of major importance but gives limited prognostic information and no prediction on the benefit of a chosen therapy (2, 3). Additional prognostic and predictive markers are needed (3). With the exception of microsatellite instability, which only concerns a minority of rectal cancers, recent genetic and molecular biology analyses of rectal cancers did not provide novel prognostic markers (4). One possible reason for this limited prognostic accuracy was the assumption, until recently, that tumor progression would be essentially a cell-autonomous process not influenced by the microenvironment (5). The paradigm could be now different as a large body of data from retrospective cohorts of human solid tumors demonstrated that the in situ immune infiltrate deeply influences the outcome of the patients (6).

We provided evidence that the type, the density, and the location of immune cells within tumor samples strongly influence the evolution of human colorectal cancers (stage I–IV; ref. 7). Thus, the adaptive immune reaction composed of T lymphocytes (CD3+) with cytotoxic (CD8+) and memory (CD45RO+) phenotype within the core of the tumor (CT) and the invasive margin (IM) is a highly significant parameter to predict recurrence and survival (7). To promote the use of this immune investigation as a routine
testing for cancer classification, we established a methodology named “Immunoscore” that provides a score based on the numeration of CD3$^+$ and CD8$^+$ lymphocytes in the CT and in the IM regions of tumors (c.f., Material and Methods for details). This classification seems to be more accurate to anticipate the outcome events than the TNM classification (8). Importantly, an independent international panel of 22 expert laboratories has started to work on large retrospective cohorts of colon cancers to promote the Immunoscore in routine clinical settings (9, 10). In a first step, it has been decided not to consider rectal tumors until a dedicated study evaluating the impact of the immune infiltrate on the tumor progression, prognosis, and response to preoperative chemoradiation therapy (pCRT) in rectal cancers was performed. This is the scope of this study. On the basis of the positive results of this study, the evaluation of the immune marker on an international multicenter study should now be initiated.

Translational Relevance
In previous publications, we demonstrated that the immune infiltrate within colorectal tumors strongly influenced the outcome of patients with colorectal cancer. We then created an "Immunoscore" to quantify the immune infiltrate. This method is currently tested worldwide on retrospective cohorts of patients with colonic cancers (22 centers; >9,000 patients) to promote the Immunoscore in routine clinical settings. Because of distinct clinicopathologic features, tumor markers, and treatment regimens when compared with colonic cancers, it has been decided not to consider rectal tumors until a dedicated study evaluating the impact of the immune infiltrate on the tumor progression, prognosis, and response to preoperative chemoradiation therapy (pCRT) in rectal cancers was performed. This is the scope of this study. On the basis of the positive results of this study, the evaluation of the immune marker on an international multicenter study should now be initiated.

Histopathologic analysis
All the hematoxylin and eosin (H&E) sections of the rectal cancers were examined by pathologists for evaluation of TNM stage, tumor differentiation, lymph node ratio (LNR) defined as the number of positive lymph nodes divided by the total number of lymph nodes examined (16), presence of tumor emboli in vascular, lymphatic, or perineural structures (VELIPI status; ref. 17), and the quality of resection (R status; ref. 4). "Downstaging" was defined as any pathologic stage (ypTNM) less than pretreatment imaging stage. Tumor regression grade (TRG) based on tumor-fibrosis ratio was determined as recommended (18).

Tissue microarray construction, staining, and analysis
For tissue samples harvested on surgical specimens, two cores were taken from CT and two cores from IM (Fig. 1A) for tissue microarray (TMA) construction as previously described (7). Slides immunostained for CD3 and CD8 (SP7 and 4B11, respectively; Neomarkers) were quantified using an image analysis workstation (Spot Browser; ALPHELYS). The "minimum P value" approach was applied to obtain the cutoff providing the best separation between the groups of patients (high vs. low) related to their disease-free survival (DFS) outcome. Accordingly, the cutoff values determined for CD3$^+$ and CD8$^+$ cell densities were 256 (Fig. 1B) and 202 cells/mm$^2$ in the CT and 144 and 50 cells/mm$^2$ in the IM, respectively.

Determination of the Immunoscore
Patients were stratified according to the "Immunoscore" ("I") ranging from 10 to 14, depending on the total number of high densities observed (CD3$^+$ cells and CD8$^+$ cells in the tumor regions; refs. 8-10). For example, I4 refers to a tumor with high densities of CD3$^+$ and CD8$^+$ cells in CT and IM regions of the tumor (4-Hi); I0 refers to tumors with...
low densities of CD3 and CD8 in both tumor regions (0-Hi; Fig. 1C).

Biopsy samples and staining

Biopsy samples were incubated for 32 minutes at 37°C with mouse monoclonal antibodies against CD3 (C8/144B; Dako; 1:50 dilution) and 20 minutes at 37°C with rabbit monoclonal antibodies against CD8 (2GV6; Ventana). The ultraView Universal DAB IHC Detection Kit (Ventana) was applied for detecting primary antibodies. High-resolution digital slides were obtained with a NanoZoomer scanner (Hamamatsu). The density of stained cells in the tumor areas was determined using the computerized image analysis system Developer XD (Definiens). Each tumor area was divided into tiles of 0.8 mm sides. The density of the immune cells stained in each biopsy was expressed as the mean density of the three most infiltrated tiles.

Statistical analysis

Parametric (Student t test) and nonparametric (Wilcoxon–Mann–Whitney test) tests were used to identify markers with a significantly different expression among patient groups. Kaplan–Meier curves were used to visualize differences between DFS and overall survival (OS). Significance among patient groups was calculated by using the log-rank test. DFS log-rank P values were corrected using the formula proposed by Altman and colleagues (19). We used a multivariate Cox proportional hazards model to determine HRs. HRs were corrected as suggested by Hollander and colleagues (20). All tests were two-sided, and a P < 0.05 was considered statistically significant. All analyses were done using the statistical software R (survival package) implemented as a statistical module in TME.db (15).

Results

Prognostic factors in patients with rectal cancer treated by primary surgery

Clinicopathologic data. The prospectively registered cohort of 111 patients with rectal cancer who underwent a primary resection of the tumor with mesorectum excision was investigated. Univariate analysis showed that TNM staging, T stage, and LNR significantly influenced DFS and OS (P < 0.05 for all comparisons). In addition, the OS was also influenced by the age of the patients, N stage, and the presence of tumor emboli (Table 1).

Impact of the immune infiltration. The densities of CD3⁺ and CD8⁺ immune infiltrates were assessed in CT and IM regions (Fig. 1A) by immunohistochemical-based TMA analyses (Fig. 1B) with image analysis software. Positive significant associations were observed between densities of CD3⁺ and CD8⁺ cells in tumor regions and clinical outcomes for DFS and OS (Supplementary Table S3). A combined analysis of tumor regions was performed. Patients with high density of a marker in both CT and IM regions were classified “HiHi”; patients with low density of such marker in both tumor regions were classified “LoLo”; patients with a high density of such marker in a single tumor region (CT or IM) were classified “Het.” HRs were 4.57 and 5.18 for CD3 and 5.88 and 6 for CD8 between patient groups (HiHi vs. LoLo) for DFS and OS, respectively (all P < 0.004 by the log-rank tests; Table 1). This combined analysis of CT plus IM regions was more efficient to discriminate patient’s outcome when compared with single region analysis (Table 1 and Supplementary Table S3). Kaplan–Meier curves illustrate the significant longer DFS (Fig. 2) and OS.
Table 1. Univariate analyses for DFS and OS among patients with rectal cancer eligible for primary surgery

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of pts (%)</th>
<th>DFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>46 (41.4)</td>
<td>70.3 (57.5–85.9)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>65–75</td>
<td>32 (28.8)</td>
<td>73 (57.7–92.4)</td>
<td>0.79 (0.32–1.99)</td>
</tr>
<tr>
<td>75–85</td>
<td>25 (22.5)</td>
<td>59.3 (39.9–89.4)</td>
<td>0.76 (0.31–1.96)</td>
</tr>
<tr>
<td>85</td>
<td>8 (7.2)</td>
<td>93.3 (58.3–100)</td>
<td>0.68 (0.39–1.22)</td>
</tr>
<tr>
<td>UICC (TNM) stage</td>
<td></td>
<td>1.62 (1.1–2.38)</td>
<td>0.0590</td>
</tr>
<tr>
<td>0–I</td>
<td>58 (52.3)</td>
<td>79 (68.7–90.9)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>II</td>
<td>27 (24.3)</td>
<td>64.9 (48.1–90.3)</td>
<td>1.3 (0.5–3.3)</td>
</tr>
<tr>
<td>III</td>
<td>16 (14.4)</td>
<td>52.5 (32.2–85.6)</td>
<td>2.5 (1.0–6.3)</td>
</tr>
<tr>
<td>IV</td>
<td>10 (9)</td>
<td>0 (NA-NA)</td>
<td>4.6 (1.0–20.81)</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td>1.72 (1.1–2.64)</td>
<td>0.0244</td>
</tr>
<tr>
<td>pTis-1</td>
<td>24 (21.6)</td>
<td>95.5 (87.1–100)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>pT2</td>
<td>41 (36.9)</td>
<td>67.2 (53.5–84.3)</td>
<td>9.71 (1.2–73.9)</td>
</tr>
<tr>
<td>pT3</td>
<td>39 (35.1)</td>
<td>53.7 (37.8–76.3)</td>
<td>12.34 (1.61–94.4)</td>
</tr>
<tr>
<td>pT4</td>
<td>7 (6.3)</td>
<td>75 (42.6–100)</td>
<td>5.37 (0.34–85.9)</td>
</tr>
<tr>
<td>N stage&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td>1.71 (0.8–2.98)</td>
<td>0.0714</td>
</tr>
<tr>
<td>N0</td>
<td>88 (79.3)</td>
<td>74.2 (64.8–85)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>N1</td>
<td>13 (11.7)</td>
<td>42.4 (20.6–87.2)</td>
<td>2.7 (1.0–6.7)</td>
</tr>
<tr>
<td>N2</td>
<td>10 (9)</td>
<td>60 (29.3–100)</td>
<td>2.06 (0.4–8.8)</td>
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<tr>
<td>LNR&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>2.13 (1.28–3.54)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>0</td>
<td>88 (81.5)</td>
<td>72.8 (63.3–83.8)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>&lt;0.33</td>
<td>10 (9.3)</td>
<td>60 (33.1–100)</td>
<td>1.54 (0.4–5.1)</td>
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<tr>
<td>0.33–0.66</td>
<td>4 (3.7)</td>
<td>66.7 (30–100)</td>
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<td>&gt;0.66</td>
<td>6 (5.6)</td>
<td>0 (NA-NA)</td>
<td>56.73 (7.78–143.75)</td>
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<td>VELIPI</td>
<td></td>
<td>1.55 (0.54–4.48)</td>
<td>0.4117</td>
</tr>
<tr>
<td>N0</td>
<td>92 (84.4)</td>
<td>71.7 (62.3–82.5)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>Yes</td>
<td>17 (15.6)</td>
<td>58.9 (34.6–100)</td>
<td>1.55 (0.54–4.48)</td>
</tr>
<tr>
<td>CD3 (CT/IM)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>2.0 (1.23–3.22)</td>
<td>0.0983</td>
</tr>
<tr>
<td>LoLo</td>
<td>7 (7.7)</td>
<td>21.4 (3.8–100)</td>
<td>4.57 (1.94–10.75)</td>
</tr>
<tr>
<td>Het</td>
<td>25 (27.5)</td>
<td>65.5 (47.8–89.9)</td>
<td>1.15 (0.92–1.44)</td>
</tr>
<tr>
<td>HiHi</td>
<td>59 (64.8)</td>
<td>71.9 (60.4–85.7)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>CD8 (CT/IM)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>2.27 (1.43–3.70)</td>
<td>0.0817</td>
</tr>
<tr>
<td>LoLo</td>
<td>13 (15.3)</td>
<td>38.9 (16.9–89.7)</td>
<td>5.88 (2.16–15.97)</td>
</tr>
<tr>
<td>Het</td>
<td>40 (47.1)</td>
<td>56.3 (41.2–76.8)</td>
<td>2.98 (1.26–7.06)</td>
</tr>
<tr>
<td>HiHi</td>
<td>32 (37.6)</td>
<td>85.7 (73.6–99.7)</td>
<td>1.0 (reference)</td>
</tr>
</tbody>
</table>

**NOTE:** VELIPI denotes the presence of vascular emboli (VE), lymphatic invasion (LI), and perineural invasion (PI), alone or in combination (information not available for 2 patients).

**Abbreviations:** LNR, lymph node ratio; NA, not applicable.

<sup>a</sup>HR corrected (20).

<sup>b</sup>log-rank P value corrected (19).

<sup>c</sup>TNM 6th edition.

<sup>d</sup>Information not available for 3 patients.

<sup>e</sup>For patients with data available on TMA analyses.

Impact of the Immunoscore. The "Immunoscore" uses the numerical density of prCD3<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in tumor regions (CT/IM) classified patients treated by primary surgery in subgroups with statistically different clinical outcomes.

- **Clin Cancer Res; 20(7) April 1, 2014**

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were as follows: 35% I4, 28% I3, 25% I2, 7% I1, and 5% I0, with an increasing risk of relapse from I4 to I0, with
the associated HRs: 1, 1.69, 2.69, 3.1, and \( P = \ldots \) respectively, for the DFS (log-rank test corrected
\( P = 0.0038 \)) and HRs of 1, 2.63, 4.45, 4 and \( P = \ldots \) respectively, for the OS (log-rank
test \( P = 0.0003 \); Supplementary Table S3). Kaplan–Meier
curves illustrating the DFS and OS times according to the
Immunoscore are shown in Fig. 3. Significant differences
between patient groups for survival times were also
observed after grouping the patients I0 and I1, which
experienced the poorest postoperative outcome (Supple-
mentary Fig. S2).

When combining the Immunoscore with the clinicopa-
thologic markers, only the Immunoscore and the lymph
node ratio (LNR) remained significant for DFS and OS in
the model after stepwise-based Cox multivariate analysis
(Immunoscore: \( P = 0.007 \) and \( P = 0.002 \); LNR: \( P = 0.04 \) and
\( P = 0.0007 \), for DFS and OS respectively; Table 2). We
then performed a Cox multivariate regression analysis by adding
tNM staging to the Immunoscore into the model. Strik-
ingly, the Immunoscore remained highly significantly
associated with DFS, whereas the TNM staging did not
reach significance. A strong impact of the Immunoscore on
the OS was also evidenced (HR of 0.62 \( P = 0.0004 \); Table
2). As a result, the Immunoscore seems to be a highly
significant prognostic factor in the group of patients treated
by primary surgery.

Is the natural immune infiltration, in patients treated
by pCRT before surgery, a prognostic factor?
To question this issue, we first investigated a historic
series of 33 patients that would be nowadays eligible for
pCRT (21), to evaluate whether the natural immune infil-
tration could influence the clinical outcome. Significant

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**Figure 2.** A, Kaplan–Meier curves for the duration in months of DFS according to T-cell (CD3\(^+\)) density evaluated in combined tumor regions (CT and IM). B, Kaplan–Meier curves for the duration of DFS according to cytotoxic T-cell (CD8\(^+\)) density evaluated in combined tumor regions (CT and IM). For each marker (CD3 and CD8), significant difference \( P < 0.005 \) was observed for DFS times between patients with high densities in the CT and in the IM (HiHi; red line), and low densities in the CT and in the IM (LoLo; black line). Patients at risk at each interval in the Kaplan–Meier survival curves for the duration of DFS are shown.
pCRT induces histologic reactions precluding the realization of an Immunoscore as the architecture of a treated tumor is deeply modified and the delineation of the analyzed tumor regions (CT and IM) is often no longer practicable.

To circumvent this issue, biopsies performed before pCRT for diagnosis were investigated for the immune infiltrate in a recent cohort of 55 patients (Fig. 4B). We asked whether the immune infiltration could predict the response to pCRT, as pCRT induces cell death forms with immunogenic potential in rectal tumors (22). The ypTNM downstaging and TRG were used as endpoints to evaluate response to pCRT (4, 18). High infiltration of CD3\(^+\) cells in tumor biopsies predominate (72% of the cases) in the subgroup of responders (complete or partial response) to pCRT (Fig. 4B), whereas 63% of the biopsies with a low infiltration of CD3\(^+\) cells belong to the group of nonresponders to pCRT (for CD3, Fisher exact test \(P = 0.015\)). The same pattern was observed for CD8\(^+\) cells (data not shown). The TRG4, 3, 2, 1, 0 evaluated on surgical specimens from patients treated by pCRT were found in 7.3%, 45.4%, 32.7%, 7.3%, and 7.3% of the cohort, respectively. The lowest infiltration of CD3 and CD8 was observed in patients TRG0, without any sign of tumor regression (data not shown). Thus, the assessment of the immune infiltrate in biopsies could help to anticipate the patient’s response to pCRT.

Discussion

Rectal cancer is a major public health issue with 80,000 new cases per year in Europe (23). Current therapeutic strategies for rectal cancers, which may strongly impact patient’s quality of life (24), are based on clinicopathologic

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### Table 2. Multivariate Cox proportional hazard analysis for DFS and OS among patients with rectal cancer eligible for primary surgery

<table>
<thead>
<tr>
<th></th>
<th>DFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Model before stepwise (stepAIC) selection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.14 (0.74–1.75)</td>
<td>0.5646</td>
</tr>
<tr>
<td>Tumor (T) stage</td>
<td>1.63 (0.95–2.8)</td>
<td>0.0750</td>
</tr>
<tr>
<td>N stage</td>
<td>0.42 (0.08–2.28)</td>
<td>0.3174</td>
</tr>
<tr>
<td>LNR</td>
<td>4.03 (0.88–18.46)</td>
<td>0.0727</td>
</tr>
<tr>
<td>VELIPI + (tumor emboli)</td>
<td>0.79 (0.18–3.45)</td>
<td>0.7564</td>
</tr>
<tr>
<td>Immunoscorea (I0 to I4)</td>
<td>0.62 (0.44–0.87)</td>
<td>0.0061</td>
</tr>
<tr>
<td>Model after stepwise (stepAIC) selection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.66 (1.25–2.22)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Tumor (T) stage</td>
<td>1.59 (0.94–2.7)</td>
<td>0.0836</td>
</tr>
<tr>
<td>LNR</td>
<td>1.88 (1.02–3.46)</td>
<td>0.0414</td>
</tr>
<tr>
<td>Immunoscorea (I0 to I4)</td>
<td>0.62 (0.44–0.88)</td>
<td>0.0069</td>
</tr>
<tr>
<td>Immunoscorea (I0 to I4)</td>
<td>1.43 (0.94–2.19)</td>
<td>0.0977</td>
</tr>
</tbody>
</table>

NOTE: All categorical covariates were transformed into numeric codes before they entered into the Cox model.

Abbreviation: AIC, Akaike information criterion.

*aLeave-one-out method. Correction using \(C = 1 – (SE[coef]/coef)^2\); heuristic shrinkage factor corrected with Hollander et al. (20).
staging systems that do not take into account biologic features of the tumor (4). There is a need for new prognostic and predictive markers to avoid under- or overtreatment in the neoadjuvant and adjuvant settings (3).

Our study was designed to address these questions. Period of inclusion is stated between 1987 and 2003 as in our institution mesorectal excision was routinely performed since 1987, and as in rectal cancer a 10-year follow-up is required for clinical studies (25). With respect to these limitations, surgical results and outcomes of our series were comparable with published data (26, 27).

We herein show that the densities of CD3\(^+\) and CD8\(^+\) lymphocytes and the associated Immunoscore (from I0 to I4) significantly correlated with DFS and OS times. These results are in line with publications showing a beneficial impact of cytotoxic T lymphocytes and the associated Th1 immune orientation in tumors of diverse origins: melanoma, head and neck, breast, bladder, urethelial, ovarian, renal, prostatic, lung, colorectal (6–8, 28–31), and few series of rectal cancer (32–34). This corpus of data strongly suggests that tumor behavior should now be considered as the result of a balance between the invasive tumor process and the response of the host of which the local immune reaction is a major component (5, 6).

We further illustrate the beneficial impact of a coordinated immune reaction in specific tumor regions (i.e., the core of the tumor and the invasive margin) to prevent recurrence and increase survival, as we observed in colon cancers (7). As a result, we demonstrate the prognostic power of the Immunoscore, which summarizes the information of the immune-cell densities in these tumor regions (8). The Immunoscore classified nearly 50% of the patients with very distinct behaviors: 35% with very a good outcome (I4) as opposed to 12% with a poor outcome (for I0 and I1). This study confirms that there is an inverse relationship between tumor invasion and the extent of immune cell infiltration (8); 90% of the patients with the highest Immunoscore I4 presented with a localized cancer (stage I–II). But importantly, 34% of the patients with a localized cancer (stage I–II) presented with an Immunoscore associated with a very poor outcome (I0–I2); conversely, 16% of the patients with an advanced rectal cancer (stage III–IV) presented with an Immunoscore associated with a very good outcome (I4); Supplementary Table S3). These data illustrate how the Immunoscore overcomes the TNM scoring system in multivariate analyses, as we observed in colon cancer (8). To reinforce the confidence on the statistical association observed, patients with poor postoperative outcome (I0 and I1) were pooled (Supplementary Fig. S2); again, the multivariate analysis showed the prognostic power of the Immunoscore.

We also show that LNR is the only parameter adding information to the Immunoscore to better predict the DFS and the OS. LNR, which evaluates the dynamic balance in multivariate analyses, as we observed in colon cancer (8). The Immunoscore classified nearly 50% of the patients with very distinct behaviors: 35% with very a good outcome (I4) as opposed to 12% with a poor outcome (for I0 and I1). This study confirms that there is an inverse relationship between tumor invasion and the extent of immune cell infiltration (8); 90% of the patients with the highest Immunoscore I4 presented with a localized cancer (stage I–II). But importantly, 34% of the patients with a localized cancer (stage I–II) presented with an Immunoscore associated with a very poor outcome (I0–I2); conversely, 16% of the patients with an advanced rectal cancer (stage III–IV) presented with an Immunoscore associated with a very good outcome (I4); Supplementary Table S3). These data illustrate how the Immunoscore overcomes the TNM scoring system in multivariate analyses, as we observed in colon cancer (8). To reinforce the confidence on the statistical association observed, patients with poor postoperative outcome (I0 and I1) were pooled (Supplementary Fig. S2); again, the multivariate analysis showed the prognostic power of the Immunoscore.

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adaptive immune responses (37). Future accurate tools predicting response to pCRT should probably take into account both the immune components and the genetic features of the tumor (38). We are currently evaluating, on a large cohort with a 10-year follow-up (24), whether the immune infiltrate in biopsies could predict DFS and OS times, as suggested by the immune investigation performed on surgical specimens of our historic cohort of 33 patients. A positive result could provide a rationale to assess the immune infiltrate in biopsies to predict responders to pCRT and to select patients achieving complete clinical tumor regression for their inclusion in prospective studies evaluating new strategies with minimal or even no surgery (24).

In conclusion, our work highlights the performance of the immune infiltration and the Immunoscore to predict times, as suggested by the immune investigation performed in biopsies to predict responders to pCRT and on surgical specimens of our historic cohort of 33 patients. A positive result could provide a rationale to assess the immune infiltrate in biopsies to predict responders to pCRT and to select patients achieving complete clinical tumor regression for their inclusion in prospective studies evaluating new strategies with minimal or even no surgery (24).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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