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Potential role of Tumor-infiltrating T-, B-Lymphocytes, Tumor-associated macrophages and IgA-secreting Plasma cells in long-term survival in the Rectal Adenocarcinoma patients

Authors:
Dmitry A. Zinovkin¹, Suheyla Y. Kose², Eldar A. Nadyrov¹, Sergey L. Achinovich³, Dmitry M. Los³, Tatyana E. Gavrilenko⁴, Dmitry I. Gavrilenko⁴, Jale Yuzugulen⁵, Md Zahidul Islam Pranjol²

¹ Department of Pathology, Gomel State Medical University, 246000 Gomel, Belarus
² School of Life Sciences, University of Sussex, Brighton, UK
³ Department of Anatomical Pathology, Gomel Regional Clinical Oncological Hospital, Gomel, Belarus
⁴ Republican Research Center for Radiation Medicine and Human Ecology, Gomel, Belarus
⁵ Faculty of Pharmacy, Eastern Mediterranean University, Famagusta, North Cyprus.

Running title:

Abstract
Aims

Many studies investigated the associations between the role of immune cells of rectal cancer microenvironment and survival during the first 5 years post-surgery. This is problematic as this disease has the potential to progress even after 5 years after relapse and infiltrating immune cells could play key roles. Therefore, this retrospective study investigates expression and roles of tumor-infiltrating T-lymphocytes (TIL-T), tumor-infiltrating B-lymphocytes (TIL-B), IgA+ plasma cells (IgA+ PC) and tumor-associated macrophages (TAM) in patients with or without progression over 5 years survival with rectal adenocarcinoma.

Main methods

Here we used immunohistochemical staining of CD3, CD20, IgA, CD68 positive cells and its detection in rectal cancer stroma. Data was analyzed using Mann Whitney U test, ROC, survival and Cox’s regression analysis.

Key findings

The number of TIL-T (p=0.0276), TIL-B (p<0.0001) and IgA+ PC (p=0.015) immune cells was significantly higher in rectal cancer stroma of patients with favorable outcome. Univariate Cox’s regression analysis revealed a predictive role of TIL-T (HR=0.482; 95% CI, 0.303 to 0.704; p<0.0001), TIL-B (HR=0.301; 95% CI, 0.198 to 0.481; p<0.0001) and IgA+PC (HR=0.488; 95% CI, 0.322 to 0.741; p<0.0001). Multivariate Cox’s regression analysis showed prognostic role of TIL-B (HR=0.940; 95% CI, 0.914 to 0.968; p<0.0001) and IgA+PC (HR= 0.985; 95% CI, 0.975 to 0.996; p=0.006) play role in long time survival.

Significance

CD20+ TIL-B and IgA+ cells have significant associations with long-term survival of patients with rectal cancer, with potential therapeutic intervention in cancer immunotherapy.

Keywords: Rectal cancer, tumor-infiltrating T-lymphocytes, tumor-infiltrating B-lymphocytes, IgA+ plasma cells, tumor microenvironment.
1. Introduction

Rectal cancer is a common malignant tumor of the digestive tract with high recurrence and mortality rates, with globally approximately 935,000 deaths in 2020 reported in the Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN). This is probably due to the complex tumor microenvironment (TME), anatomical features of the rectum and a lack of early diagnosis of the disease. The tumor microenvironment is comprised of immune cells with local infiltration of cancer stromal cells and their secreted active mediators, proteases and tumor cells [1].

With the growing tumors, the immune surveillance is enhanced, from natural killer (NK) cells of the innate immunity to the lymphocyte of the more long-term, adaptive immune system. The adaptive immune response is orchestrated by the antigen-specific T- and B- lymphocytes that infiltrate the stroma of the TME. Tumor-infiltrating T-lymphocytes (TIL-T) such as CD4+ and CD8+ cells are known combaters in anti-tumor immunity which can inhibit cancer growth. For instance, CD8+ T cells induce direct killing of cancer cells [2]. TIL-T and their stroma density are known to exhibit prognostic values in various types of solid tumors such as breast, gastric and laryngeal cancers [3–5] and are associated with the expression of certain biomarkers, which could serve as therapeutic targets [6]. The role of tumor-infiltrating B lymphocytes (TIL-B), however, remains less explored and in matters of prognostic importance, consensus has yet to be reached [2]. For instance, the regulatory mechanisms of TIL-B in cancer progression remain largely unknown. The release of antibodies and cytokine interleukin-10 might be involved in TIL-B-mediated tumor immunity [7], however its role in disease progression remains to be elucidated.

In healthy intestinal mucosa, plasma cells play an essential role in maintaining homeostasis by producing antibodies, especially IgA which plays a stable role in the regulation of the gastrointestinal neuroendocrine-immune system network [8]. A role of IgA+ plasma cells (IgA+-PC) in patients’ survival is now known only for breast cancer [7]. Cells of innate immunity such
as tumor-associated macrophages (TAMs) represent the most abundant immune cells within the TME and have been associated with adverse outcomes in patients with different types of cancers [9–11] IgA⁺-PC and TAM in patients with or without progression of rectal adenocarcinoma. Due to the fact that this disease has the potential to progress even after 5 years after relapse [12], we hypothesize that the stromal density of TIL-T, TIL-B, IgA⁺-PC and TAM could contribute to the long-term survival of patients with rectal adenocarcinoma.

2. Materials and Methods

Informed consent was taken from all patients included in this study. Ethical approval was obtained and reviewed by the Institutional Review Board, Gomel, Belarus (protocol №14 from 12.06.2020).

2.1. Patients

This retrospective cohort study involved patients with rectal adenocarcinoma who were treated in the Gomel Regional Clinical Oncological Hospital. The criteria of inclusion were a presence of rectal adenocarcinoma, clinical stage I-III of the disease, an absence of malignant tumors of other localizations during life, survival more than 30 days after surgical resection of the tumor. The exclusion criteria for the study were as follows: non-rectal cancers, clinical stage IV, a presence of synchronous and metachronous malignances, death during the first 30 days after surgical resection of tumor. Clinical stage IV was excluded from this study as a small number of patients had received palliative treatment.

A total of 155 out of 739 cases of rectal adenocarcinomas were determined to be eligible for inclusion in the study. Selected cases were divided into two groups; favorable and unfavorable outcome. The favorable outcome group consisted of 68 patients who showed no progression of rectal cancer during the period of observation. The group with unfavorable outcome was formed of 87 patients who suffered from progression of rectal carcinoma during observation. We
determined the role of immune cells infiltration in patients with and without radiochemotherapy. The average observation period was 7.5 years post-surgery. Patients from both groups were diagnosed and received treatment according to the protocols of treatment of colorectal cancers approved by the Ministry of Healthcare of Republic of Belarus. The clinical characteristics of patients are presented in Table 1.

2.2. Immunohistochemistry

3-4µm thick sections were made from paraffin blocks using a Microm HM 304E rotor microtome (Thermo Scientific, Germany). Next, the sections were mounted on Thermo Super-Frost poly-L-lysine coated slides (Thermo Scientific, Germany). Antigen retrieval was performed using a microwave. The sections were then allowed to cool and endogenous peroxidase blocking was performed in 5% hydrogen peroxide. The nonspecific antibody binding was blocked by 5% casein. Sections were washed and incubated in a moist chamber at room temperature with corresponding primary antibodies to CD3 (ready-to-use; Dako, Denmark), CD20 (ready-to-use; Dako, Denmark), IgA (ready-to-use; Dako, Denmark) and CD68 (ready-to-use; Dako, Denmark), followed by incubation with secondary anti-mouse/rabbit HRP antibodies. The visualization of product reaction was performed using 3,3-diaminobenzidine (DAB) staining for 5 minutes, followed by Mayer’s hematoxylin counterstaining [13]. In this study, the following primary antibodies were used for detection: anti-CD3 (TIL-T cells), anti-CD20 (TIL-B), anti-CD138 (IgA+ PCs) and anti-CD68 (TAM). The count of positive immune cells was performed in 10 non-overlapping (×400 magnification) high-power fields (HPFs) in stroma using ImageJ (NIH, USA) and HumaScope Premium LED (Human, Germany). Then number of the cell in 1 HPF was converted in number of cells/mm².

2.3. Statistical analysis

Mann-Whitney U test was used to compare the study groups. A threshold criterion was evaluated by ROC-analysis. The cut off criteria revealed by ROC-analysis was validated using Fisher’s exact
3. Results

3.1. Tumor-infiltrating T-lymphocytes

A dense TIL-T cell-infiltrate was observed in the desmoplastic stroma near the cancerous glands in the area of invasion (Figure 1A). The median of TIL-T cells in favorable prognosis group was significantly higher (p=0.0276) than in unfavorable prognosis group (Figure 2A). On the basis of the ROC-analysis, a threshold of 66.3 cells/mm² for TIL-T was adopted as the best differentiating value between patients with different clinical outcomes, e.g. with 66.3% specificity and 51.4% sensitivity (area under the curve was 0.60; p=0.0281). Validation of the threshold criteria revealed that likelihood ratio, sensitivity and specificity were 2.095, 78.57% (95%CI 52.41% to 92.43%), and 62.50% (95%CI 38.64% to 81.52%) respectively. Log-rank test revealed a significantly higher (p=0.0004) survival of patients with TIL-T cells infiltration than the threshold criteria (Figure 3A).

3.2. Tumor-infiltrating B-lymphocytes

Large groups of TIL-B lymphocytes were situated in the lymphoid cell infiltrate in the area of rectal cancer invasion (Figure 1B). Statistically significant increase in number of CD20⁺ TIL-B cells (p<0.0001) were found between the two patient groups (Figure 2B). In ROC analysis, the area under the curve was 0.71 (p<0.0001), where the sensitivity, specificity and threshold criteria
were 56.3%, 83.4%, and 12.4 cells/mm² respectively. Validation of the threshold criteria revealed that likelihood ratio, sensitivity and specificity were 7.429, 92.86% (95%CI 68.53% to 99.63%) and 87.50% (95%CI 63.98% to 97.78%) respectively. A survival analysis revealed statistically significant differences (p<0.0001) (Figure 3B) between the two groups.

3.3. IgA-positive plasma cells

In the favorable group, IgA⁺ PC were present in small groups of cells in the stroma (Figure 1C). Statistically significant differences in the number of IgA⁺ PC (p=0.015) were observed (Figure 2C) between the two groups. The area under the curve from ROC-analysis of number of the IgA⁺ PC was 0.59 (p=0.0525). The sensitivity, specificity and threshold criteria were 64.4%, 60.3%, and 38.7 cells/mm² respectively. Validation of the threshold criteria revealed that likelihood ratio, sensitivity and specificity were 3.810, 71.43% (95%CI 45.35% to 88.28%) and 81.25% (95%CI 56.99% to 93.41%) respectively. Log-rank test showed a significantly (p=0.001) higher survival rate, with a higher number of IgA⁺ PCs than the threshold criterion (Figure 3C).

3.4. Tumor-associated macrophages

TAM infiltration in both groups was predominantly situated near the microvessels in the rectal cancer stroma (Figure 1D). We did not observe any statistically significant differences in the number of TAM between the two groups (Figure 2D). On the basis of the ROC-analysis, a threshold of 101.5 cells/mm² for CD68-positive TAM was adopted as the best differentiating value between patients with different clinical outcomes, with 81.6% specificity and 29.4% sensitivity (area under the curve was 0.52; p=0.650). Validation of the threshold criteria revealed that likelihood ratio, sensitivity and specificity were 1.469, 64.29% (95%CI 38.76% to 83.66%) and 56.25% (95%CI 33.18% to 76.90%) respectively. Survival analysis didn’t revealed a statistically significant difference (p=0.248) (Figure 3D).

3.5. Role of radiochemotherapy
Potential role of radiotherapy in survival was analyzed using Mann-Whitney test which revealed no statistically significant differences in TIL-T, TIL-B, IgA+PC and TAM cells infiltration in patients with and without radiochemotherapy (Supplement 1).

3.6. Univariate and Multivariate Cox’s regression analysis

A univariate analysis revealed that all parameters except TAM play a role in DFS of patients with rectal adenocarcinoma (Figure 4A). However, our multivariate Cox proportional hazard regression analysis demonstrated that only TIL-B (HR=0.940; 95% CI, 0.914 to 0.968; p<0.0001) and IgA+ PC (HR= 0.985; 95% CI, 0.975 to 0.996; p=0.006) were significantly associated with DFS (Figure 5B)

4. Discussion

The role of the immune system in cancer remained unappreciated for many decades. This is largely due to the efficient immunosuppressant effect of tumors and the TME. It is known that tumors are able to evade immune surveillance by activating negative regulatory pathways (also called checkpoints) that are associated with immune homeostasis or adopting cellular and molecular features, including immune cells, to actively escape the immune system [14–17] stroma, may play a role in the prognosis, potentially predicting the outcome of the disease [10]. Therefore, in this study we investigated the role of TIL-T, TIL-B, IgA+ PCs and TAMs in anticancer immune response and DFS.

T cell-mediated adaptive immune response plays an important role in cancer progression. A higher density of TIL-T has been reported to be associated with better prognosis in patients with various types of cancers, including colorectal cancer [18]. Our study also showed higher infiltration of rectal adenocarcinoma margin by TIL-T in patients with favorable outcome compared to the unfavorable group. Migration of T-lymphocytes from the circulation to the tumor site implies that the host immune system is capable of initiating an anti-tumor response. However, changes that occur as a result of cellular mutations in the pro-inflammatory hypoxic environment within tumors eventually create an immunosuppressive microenvironment that prevents tumor
eradication by TIL-T [19]. As such, we observed a decreased density of TIL-T cells in patients with unfavorable prognosis which may indicate a tumor-induced anti-immune response. [20,21] but not in multivariate Cox’s regression analysis. This is possibly because of an indirect activation of TIL-T in response to chemo and radiotherapy that plays a role in DFS in cases of rectal cancer with high TIL-T cell infiltration [22].

It has been shown that TIL-B can differentiate into different subtypes under the influence of the TME, while different TIL-B subtypes play a dual role in the anti-tumor immunity by secreting antibodies, presenting antigens, and secreting cytokines [23]. In general, TIL-B cells are commonly activated and have memory phenotypes and can activate tumoricidal T cells to increase cancer cell killing due to their effectiveness in antigen presentation and co-stimulation [24]. Our study showed an increase in the number of TIL-B in the cancer stroma of patients with favorable prognosis which may play an important anti-tumor role [25,26]. For instance, Berntsson et al (2016) showed a correlation between TIL-B cells and an improved favorable prognosis in patients with colorectal cancer which may suggest that the presence of B cells in the cancer stroma is a marker of a “good” overall immune status of the patient [27]. Interestingly, Edin et al. (2019) reported a prognostic role for CD20-positive TIL-B cells in the survival of patients with colorectal cancers using multivariate Cox’s regression, confirming our findings [2].

The most well-known function of IgA is that it provides a passive immunity through immune exclusion, pathogen neutralization, and antigen excretion, particularly at mucosal sites such as the gastrointestinal tract [28]. In the intestine, IgA is produced in large quantities in dimeric form by plasma cells in the lamina propria and play a role in inflammatory conditions of large intestine and colorectal cancer [29]. According to Muthuswamy et al. (2013), a low IgA secretion at the tumor site could lead to a reduced local barrier function and a higher risk of bacterial penetration into the tissues [30]. IgA+ PCs may reduce bacteria-induced inflammation at the tumor site, and that a lack of IgA at the tumor site may lead to an increase in local inflammation to promote tumor progression [31]. This could explain the high level of IgA+ PC infiltration in rectal
adenocarcinoma in our observed patients’ cases with favorable prognosis. In our study, both univariate and multivariate regression analysis revealed a potential role for IgA+ PC in long-term survival. A similar finding was reported by Benckert and colleagues, where immunized colorectal cancer patients with higher IgA titers showed better survival, although the relationship was also significant in multivariate regression analysis [32].

Macrophages are one of the immune cell populations frequently found in colorectal tumors and a high macrophage infiltration has been associated with both favorable and worse prognosis [33]. Others reported that a high CD68+ macrophages infiltration resulted in better survival in colorectal cancer patients [34,35], while others revealed that TAM mainly exhibited a M2-like [36–38] study revealed no statistically significant difference in the number of TAM, and their role in survival could be associated with an imbalance of M1 and M2 TAM in these patients. The role of TAM in the prognosis of rectal adenocarcinoma should be investigated with a focus on M1 and M2 subtypes of TAM.

The favorable impact of TIL-B infiltration in our patients’ survival may be the antigen presenting function of B cells which demote cancer progression via antibody-dependent cellular cytotoxicity. Another explanation may be that the previously presence of TIL-B represents patients’ overall immunity and that those patients who are able to bring forth an immune response may pre-selectively be in a better overall condition [27]. TIL-B has also been shown to kill tumor cells by secreting granzymes, an antibody-independent pathway [39]. Following antigen exposure and T cell licensing, B cells differentiate into potent antibody-secreting plasma cells, which no longer express CD20 [40]. These plasma cells secrete IgA which crosses tumorepithelial cells by transcytosis and protects IgA from proteolytic degradation, activating T-cell-mediated anticancer immune response [41]. We report that a high infiltration of IgA+ PC is associated with better prognosis in rectal cancer, which has recently been shown for breast cancer patients [42]. This overexpression of IgA+ PCs could be utilized in developing immunotherapy targeting rectal cancer.
In our analysis on the effect of radiochemotherapy, we demonstrated that patients who underwent such treatment revealed no differences in the immune cell infiltration. This indicates that the potential roles of these types of infiltrating immune cells in long term survival are independent of these treatment regiments. This is possibly due to parameters such as tumor size, high levels of CEA and MRI-detected extramural vascular invasion which potentially have higher influence on the radiochemoresistance of rectal cancer than immune cells [43].

In comparison to previous studies, our work revealed a potential role of CD20+ TIL-B and IgA+ plasma cells in long-term survival. Most studies reported a 5-year survival, however, it is well known that rectal cancer could progress even after this period. In our study we showed associations between these cells and long-term survival post-5-year period. This signifies the importance of following up with patients after relapse and demonstrates the necessity to assess the infiltrated immune cells dynamic in the microenvironment which may be utilized to predict patients’ long-term survival and treatment.

In conclusion, this study presents potential associations of CD20+ TIL-B and IgA+ plasma cells in survival of patients with rectal adenocarcinoma. The results from this retrospective study should be further investigated for cellular and molecular interactions and mechanisms for potential development of cancer immunotherapy.

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Conflict of interest

The authors declared that they have no conflict of interest.
**Ethical approval**

This study was performed according to the ethical standards of the local Ethics Committee of Gomel State Medical University and in compliance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

**Informed consent**

Informed consent was obtained from all participants included in the study.

**References**


**Figure 1.** Immunohistochemical staining immune cells: A. Dense CD3$^+$ T-lymphocytes infiltrate cancer stroma of rectal cancer in the tumor invasive margin C. CD20$^+$ B-cells forming dense round form infiltrate in favorable outcome group; C. Big group of IgA$^+$ plasma cells infiltrating cancer stroma near tumor glands; D. Rectal cancer stroma mildly infiltrated by CD68$^+$ tumor-associated macrophages. Magnification: ×100.

**Figure 2.** Boxplot diagrams presenting the comparing of favorable and unfavorable outcome groups according the number of: A. CD3$^+$ T-lymphocytes; B. CD20$^+$ B-lymphocytes; C. IgA$^+$ plasma cells; D. CD68$^+$ macrophages.

**Figure 3.** Kaplan–Meier survival curves of rectal adenocarcinoma cases separated by ROC-analysis revealed levels of: A. tumor-infiltrating CD3$^+$ T-lymphocytes; B. tumor-infiltrating CD20$^+$ B-lymphocytes; C. tumor-infiltrating IgA$^+$ plasma cells; D. CD68$^+$ tumor-infiltrating macrophages.

**Figure 4.** Hazard ratios according: A. univariate Cox’s regression analysis; B. multivariate Cox’s regression analysis; B. multivariate Cox’s regression analysis.