

Morphological features of the spatial distribution of T-lymphocytes in colorectal cancer with metastases to regional lymph nodes

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Aim. To determine the nature, intensity, and spatial distribution of CD3⁺ and CD8⁺ T-lymphocytes in colorectal cancer (CRC) with regional lymph node metastases and to identify immune phenotypes by cluster analysis.

Materials and methods. An immunohistochemical study was performed with antibodies to CD3⁺ and CD8⁺ on surgical specimens from 27 patients with histologically verified CRC and metastases to regional lymph nodes. The tumor center and invasive margin were analyzed for the localization and distribution pattern of CD3⁺ and CD8⁺ cells in a standardized field of view (SFV) of 100 × 100 μm². Quantitative data were expressed as median and interquartile range (IQR). Spatial heterogeneity was assessed using the quartile coefficient of dispersion (QCD). Differences between tumor compartments were evaluated with the Wilcoxon signed-rank test. Hierarchical cluster analysis was performed using Ward's method after data standardization.

Results. The median CD3⁺ lymphocytes density was significantly higher at the invasive margin than in the tumor center (7.30 [5.55; 13.55] vs. 4.80 [3.32; 5.18] p < 0.001). The same pattern held for CD8⁺ lymphocytes (3.40 [2.60; 4.00] vs. 1.70 [1.20; 2.50] cells per SFV; p < 0.001). The highest spatial variability was observed for CD3⁺ cells at the invasive margin (QCD = 0.419). Cluster analysis identified three immune phenotypes: immune-cold (44.44 %), immune-excluded (37.04 %), and immune-hot (18.52 %).

Conclusions. Colorectal cancer with regional lymph node metastases exhibits pronounced spatial heterogeneity of T-cell infiltration and preferential accumulation of CD3⁺ and CD8⁺ lymphocytes at the invasive margin. Immune phenotyping based on spatial CD3⁺/CD8⁺ analysis enables biologically meaningful tumor stratification and may inform prognosis and personalize immunotherapy selection.

Keywords:

colorectal cancer, lymph node metastasis, tumor-infiltrating CD3, CD8-lymphocytes, spatial heterogeneity of immune infiltration.

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Морфологічні особливості просторового розподілу Т-лімфоцитів при колоректальному раку з метастазами в регіонарні лімфатичні вузли

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Мета роботи – визначити характер, інтенсивність і просторовий розподіл CD3⁺ і CD8⁺ Т-лімфоцитів при колоректальному раку (КРР) із метастазами в регіонарні лімфовузли та визначити імунні фенотипи на основі кластерного аналізу.

Матеріал і методи. Проведено імуногістохімічне дослідження з антитілами до CD3⁺ і CD8⁺ операційного матеріалу від 27 пацієнтів з КРР і метастазами в регіонарні лімфатичні вузли. Досліджено ділянки центра пухлини та інвазивного краю, в яких аналізували локалізацію і характер розподілу CD3⁺ і CD8⁺ клітин в умовно стандартизованому полі зору площею 100 × 100 мкм². Кількісні дані наведено як медіану та міжквартильний розмах (IQR). Просторову неоднорідність оцінено за квартильним коефіцієнтом дисперсії (QCD). Для порівняння показників між центром пухлини та інвазивним краєм застосовано критерій Вілкоксона. Ієрархічний кластерний аналіз виконано методом Варда після стандартизації даних.

Результати. Медіанна щільність CD3⁺ лімфоцитів статистично значущо вища на інвазивному краї порівняно з центром пухлини (7,30 [5,55; 13,55] і 4,80 [3,32; 5,18] клітин на поле зору; p < 0,001). Аналогічну закономірність виявлено для CD8⁺ лімфоцитів (3,40 [2,60; 4,00] і 1,70 [1,20; 2,50] клітин на поле зору; p < 0,001). Найбільшу просторову варіабельність визначено для CD3⁺ клітин на інвазивному краї пухлини (QCD = 0,419). За результатами кластерного аналізу розрізняли три імунні фенотипи: immune-cold (44,44 %), immune-excluded (37,04 %) та immune-hot (18,52 %).

Висновки. Колоректальний рак із метастазами у регіонарні лімфатичні вузли характеризується просторовою неоднорідністю Т-клітинної інфільтрації та переважним накопиченням CD3⁺ і CD8⁺ лімфоцитів на інвазивному краю пухлини. Аналіз просторової організації імунного інфільтрату дає змогу ідентифікувати біологічно відмінні імунні фенотипи, може бути використаний для прогнозування перебігу захворювання і персоналізації імунотерапевтичних підходів.

Ключові слова:

колоректальний рак, метастази в лімфатичні вузли, пухлино-інфільтруючі CD3-, CD8-лімфоцити, просторова гетерогенність імунної інфільтрації.

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Colorectal cancer (CRC) remains one of the leading causes of cancer incidence and mortality worldwide, ranking third in prevalence and second in mortality among malignant neoplasms [1,2]. Modern studies show that the course and prognosis of CRC are determined not only by its morphological characteristics but also substantially by the features of the tumor immune microenvironment [3,4]. Over recent decades, convincing evidence has accumulated that the intensity and spatial distribution of tumor-infiltrating lymphocytes are independent prognostic factors in CRC [5,6]. Of greatest importance is the density of CD3⁺ and CD8⁺ lymphocytes in the tumor center and at its invasive margin, as this topographic assessment more accurately reflects the antitumor immune response than total lymphocyte counts alone [7].

CD3⁺ is a pan-T-cell marker reflecting the total T-cell infiltrate and allowing assessment of the overall adaptive cellular immune response intensity. Spatial analysis of CD3⁺ cells (intratumoral, peritumoral and stromal localization) is important, because different infiltration patterns are associated with different tumor progression. It has been shown that a high density of CD3⁺ lymphocytes in the tumor center and at the invasive margin was associated with a reduced risk of recurrence and better overall survival [8].

Conversely, insufficient intratumoral infiltration may indicate the formation of an immunosuppressive microenvironment. CD8⁺ is a cytotoxic T-lymphocyte marker and a key effector of antitumor immunity in the tumor microenvironment. In studies, CD8⁺ cells are evaluated both by total number and by spatial distribution [4]. This spatial-quantitative assessment underpins the “immune context” concept – a generalized characteristic of the immune tumor microenvironment and explains why the density of CD8⁺ infiltrate often correlates with the clinical course of the disease. A recent study has shown, that CD8⁺ lymphocyte density at the invasive margin provides independent prognostic information: cancer-specific survival was superior in patients with high CD8⁺ density both in the primary cohort and during independent validation [9].

According to the pattern of CD8⁺ T-cell infiltration, distinct tumor immune phenotypes have been described [10]. The immune-hot phenotype is characterized by a high density of CD8⁺ cells both in the tumor center and at the invasive margin, corresponding to active antitumor immunity and a more favorable prognosis. The immune-cold phenotype is characterized by low T-cell infiltration throughout the tumor, indicating impaired immune recognition and a worse prognosis. The immune-excluded phenotype is defined by accumulation of immune cells in the peritumoral stroma without penetration into tumor nests, creating a microenvironmental barrier effect.

Particular attention is directed to immune infiltration in relation to the depth of tumor invasion – in the mucosa, the muscularis propria, and at the interface with adipose tissue. Spatial heterogeneity of T-cell infiltration may reflect the staging of the immune response and the progression of the tumor process. In 2006, the Immunoscore methodology (immune infiltration index of the tumor) was introduced as a quantitative index of intratumoral immune infiltration for predicting recurrence risk. This methodology was subsequently validated by an international consortium [11] and was based on determining the density of lymphocytes infiltrating the tu-

mor, which and demonstrated superior prognostic accuracy for patient survival compared with classic TNM staging.

However, despite a substantial body of research on the role of CD3⁺ and CD8⁺ T-lymphocytes in CRC [12,13], a number of aspects of the local antitumor immune response remain insufficiently studied. In particular, the morphological analysis of the spatial organization of the T-cell infiltrate in CRC with metastatic involvement of regional lymph nodes has received limited attention.

Aim

The aim of the study is to determine the nature, intensity, and spatial distribution of CD3⁺ and CD8⁺ T-lymphocytes in colorectal cancer with regional lymph node metastases, and to identify immune phenotypes based on cluster analysis.

Materials and methods

The study included surgical specimens from 27 patients with histologically verified CRC in whom histological examination confirmed revealed in regional lymph nodes. Histological evaluation identified well-differentiated (n = 2), moderately differentiated (n = 7), and poorly differentiated adenocarcinomas (n = 18). Surgical specimens were fixed in 10 % neutral buffered formalin for 24 hours with subsequent standard processing and paraffin embedding. Serial sections 3–4 µm thick were prepared from paraffin blocks, stained with hematoxylin and eosin for routine histological assessment. An immunohistochemical (IHC) study was performed using monoclonal antibodies to CD3⁺ and CD8⁺. T-lymphocytes were labeled in paraffin sections using monoclonal antibodies Mo a-Hu CD8⁺ (clone SP16), CD3⁺ clone (EP41) and the DAKO EnVision visualization system (all reagents from DAKO, Denmark).

Deparaffinization and rehydration of sections were performed by the standard method. Antigen retrieval was carried out by heat-induced epitope retrieval in a buffer solution according to the manufacturer's protocol. After blocking endogenous peroxidase activity, primary antibodies were applied, followed by incubation with a secondary horse-radish peroxidase-conjugated visualization system. The immunoreaction was visualized using 3,3'-diaminobenzidine (DAB); nuclear counterstaining was performed with hematoxylin. Positive and negative controls were included in each staining series in accordance with IHC quality standards.

Morphometric analysis was performed on tissue regions free of necrotic changes or hemorrhages in two compartments: the tumor center (within tumor nodules) and the invasive margin (defined as the peritumoral zone encompassing the tumor invasion front and adjacent stromal tissue). To characterize the immune response, resection margins without evidence of tumor growth were additionally examined as a reference (non-neoplastic) control.

Morphological and morphometric evaluations of the specimens were performed by light microscopy using a Carl Zeiss Axio Scope A1 microscope (Germany) equipped with a Jenoptik Progres Gryphax® SUBRA series camera (Germany). In each case, 10 fields of view were analyzed using the Videotest – Morphology 5.2.0.158 software. The localization and distribution pattern of CD3⁺ and CD8⁺ cells were analyzed in the histological specimens. The density

of CD3⁺ and CD8⁺ immunopositive cells was expressed as the number of cells per standardized field of view (SFV) with an area of 100 × 100 μm².

Statistical analysis was performed using non-parametric methods. Quantitative evaluation of CD3⁺ and CD8⁺ lymphocytic infiltration was conducted separately in the tumor center and at the invasive margin. In each case, the analysis was carried out across 10 standardized fields of view, and the results were presented as median and interquartile range [Q1; Q3].

To assess parameter variability and the spatial heterogeneity of immune infiltration, the interquartile range (IQR) was determined and calculated as the difference between the third and first quartiles (IQR = Q3 – Q1). Additionally, the quartile coefficient of dispersion (QCD) was calculated using the formula: $QCD = (Q3 - Q1) / (Q3 + Q1)$.

The QCD values were utilized as a measure of the spatial heterogeneity of immune cell distribution, independent of the influence of extreme values. QCD < 0.10 was interpreted as low spatial heterogeneity, 0.10–0.30 as moderate, and > 0.30 as high heterogeneity.

Comparison of CD3⁺ and CD8⁺ lymphocyte densities between the tumor center and the invasive margin was performed using the Wilcoxon signed-rank test. Results were considered statistically significant at $p < 0.05$.

To characterize the relationship between total T-cell infiltration and the cytotoxic T-cell response, the CD3⁺/CD8⁺ ratio was calculated separately for the tumor center and the invasive margin based on the median values of the respective parameters.

To identify natural tumor groups based on immune microenvironment characteristics, hierarchical cluster analysis was performed. The analysis included CD3⁺ and CD8⁺ densities in the tumor center and at the invasive margin, standardized to Z-scores (mean = 0, SD = 1) in Microsoft Excel. The resulting standardized feature matrix was used for further hierarchical cluster analysis using Ward's linkage method via Euclidean distance in the Statistica 12 program.

Results were visualized as a dendrogram; clusters were assigned to immune-hot, immune-cold, and immune-excluded phenotypes based on their median CD3⁺ and CD8⁺ density profiles.

Results

Immunohistochemical examination of the colorectal mucosa at tumor-free resection margins revealed moderate physiological expression of CD3⁺, corresponding to the normal distribution of T-lymphocytes within the intestinal wall. CD3⁺-positive cells appeared as small mononuclear elements with distinct membranous-cytoplasmic staining, localized predominantly periglandularly around the crypts and occasionally intraepithelially between crypt enterocytes. The highest density of CD3⁺ cells was observed in the upper regions of the lamina propria; fewer cells were found in the basal regions of the crypts. Isolated cells were localized within the muscularis mucosae. Cell distribution was relatively uniform, without the formation of dense aggregates or pronounced diffuse infiltration. The cell density per SFV was 5.5 (5.0; 8.0). In areas with chronic inflammation, frequently observed in CRC patients, the CD3⁺ cell density was increased, and distribution became uneven. CD3⁺ cells were

not detected within the muscularis propria of the intestine in any examined case.

The distribution of CD8⁺ cells within the lamina propria exhibited a similar pattern, although their count was significantly lower at 2.5 (1.5; 3.0) per SFV, with substantial inter-case variability.

In CRC, both intratumoral and peritumoral components of T-cell infiltration were identified. T-lymphocytes were localized between tumor cells, occasionally penetrating glandular structures; however, their density within the stroma was typically much higher than their intratumoral density. Weakly expressed or moderate, diffuse, and relatively uniform infiltration of CD3⁺ cells was observed in the tumor center. Single cells predominated; small groups of immunopositive cells were less frequent, forming minor clusters around tumor glands and more prominent aggregations in the perivascular space (Fig. 1). The cell density in the tumor center was 4.80 (3.32; 5.18) cells per SFV.

The stroma in the tumor center was weakly or moderately developed, represented by loose connective tissue with signs of desmoplastic reaction. The majority of immunopositive cells were localized in the stroma peritumorally. Moderate density of CD3⁺ cells was determined in connective tissue layers, where cells were located mainly diffusely, sometimes in minor clusters and separated individual tumor glands. With significant desmoplastic changes, focal areas of high CD3⁺ T-lymphocyte density were observed around tumor complexes (Fig. 2), while within tumor complexes only isolated cells were present. The density of CD3⁺ cells at the invasive margin was 7.30 (5.55; 13.55) cells per SFV. Spatial heterogeneity analysis demonstrated moderate variability of CD3⁺ cells in the tumor center (QCD = 0.219).

The density of CD8⁺ T-lymphocytes in the tumor center was determined at 1.70 (1.20; 2.50), corresponding to a low level of infiltration (Fig. 3); however, cluster analysis revealed the presence of different types of immune microenvironment within the tumor, and accordingly, the median density of CD8⁺ T-lymphocytes also varied across different clusters. Areas with an almost complete absence of CD8⁺ cells, zones with CD8⁺ cells confined to the stroma without penetration into tumor complexes, and foci of increased infiltration density were all identified. A pronounced spatial heterogeneity of CD8⁺ T-cell infiltration was thereby confirmed (QCD = 0.351); the cytotoxic component was reduced and spatially disorganized.

The CD3⁺/CD8⁺ ratio in the tumor center was 2.30 (1.95; 3.18), indicating a relative deficit of cytotoxic T-lymphocytes within the total T-cell population. The obtained results suggest a dissociated T-cell response, characterized by preserved total T-cell infiltration with a reduced cytotoxic component and moderate spatial heterogeneity (QCD = 0.250).

The density of CD3⁺ T-lymphocytes at the invasive CRC margin demonstrated a moderately high level (7.30 [5.55; 13.55] cells per SFV) with pronounced spatial heterogeneity (QCD = 0.419). The IQR in this group was 8.00, substantially exceeding the IQR values in other groups (range 1.30–1.86). The density of CD3⁺ cells was markedly increased in some areas, indicating a local immune response within the tumor invasion zone (Fig. 4). Overall, the invasive margin showed significantly higher CD3⁺ infiltration than the tumor center, suggesting more active immune interaction

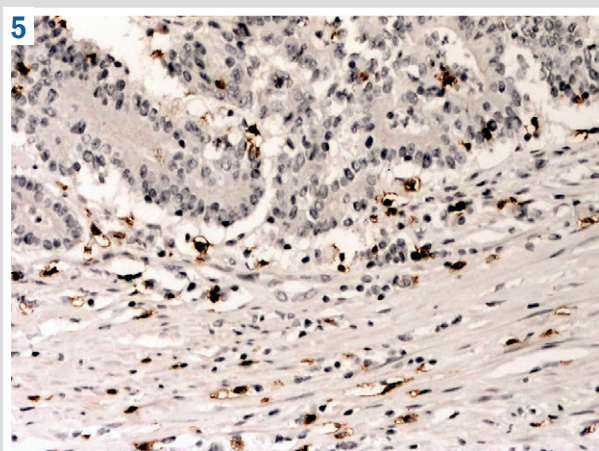
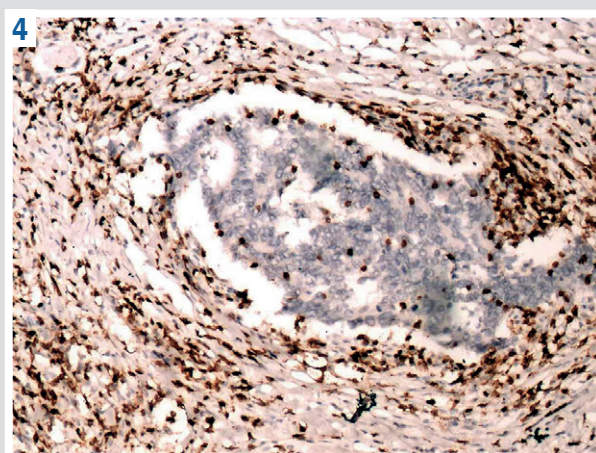
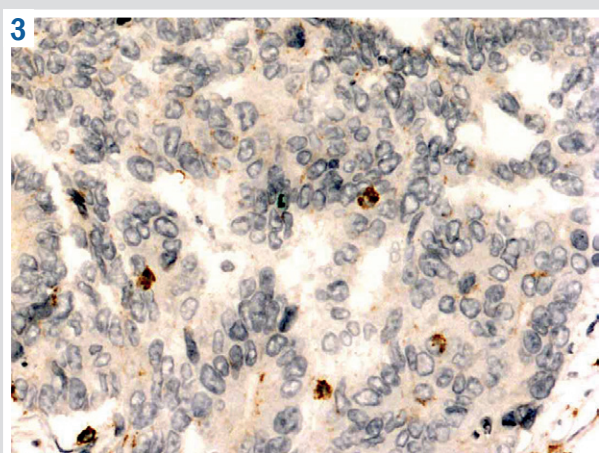
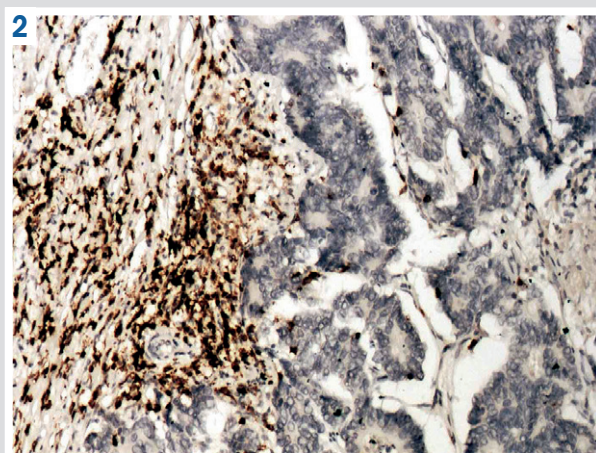
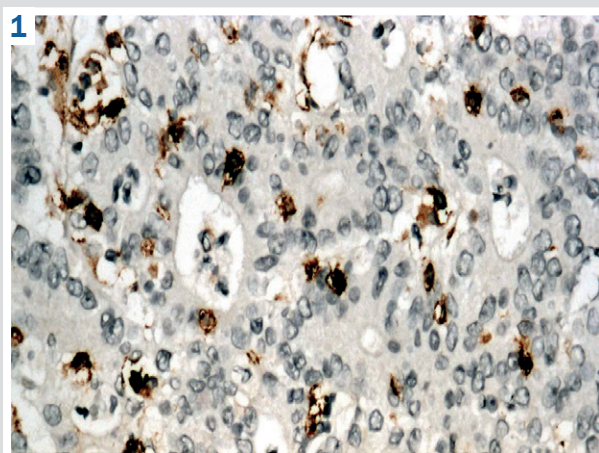


Fig. 1. CD3⁺ expression in the tumor center. IHC. Mag. ×200.

Fig. 2. High density of CD3⁺ lymphocytic infiltration in desmoplastic stroma around tumor glands. IHC. Mag. ×100.

Fig. 3. CD8⁺ expression in the tumor center. IHC. Mag. ×400.

Fig. 4. Density of CD3⁺ T-lymphocytes at the invasive margin of CRC. IHC. Mag. ×100.

Fig. 5. Low level of CD8⁺ lymphocytic infiltration at the invasive tumor margin. IHC. Mag. ×200.

in this compartment; however, cell penetration into tumor complexes was extremely limited.

The density of CD8⁺ T-lymphocytes at the invasive CRC margin was 3.40 (2.60; 4.00) cells per SFV, corresponding to low-to-moderate cytotoxic infiltration.

Across all cases, a relatively lower density of CD8⁺ effector T-lymphocytes compared with CD3⁺ cells was observed, and no dense, continuous accumulation of effector cells was identified at the tumor–stromal interface (Fig. 5). The CD3⁺/CD8⁺ ratio was 2.27 [1.74; 3.08], indicating a predominance of the total T-cell infiltrate over the cytotoxic effector component,

suggesting limited efficacy of the antitumor immune response at the invasive tumor margin. The spatial heterogeneity index indicated moderate spatial non-uniformity for the CD8⁺ cell distribution at the invasive margin (QCD = 0.212).

Based on the density of CD3⁺ and CD8⁺ lymphocytes in the tumor center and the invasive margin, hierarchical cluster analysis using Ward's method identified specific features of the immune microenvironment in CRC with regional lymph node metastases. This analysis yielded three clusters differing in the intensity and spatial organization of T-cell infiltration.

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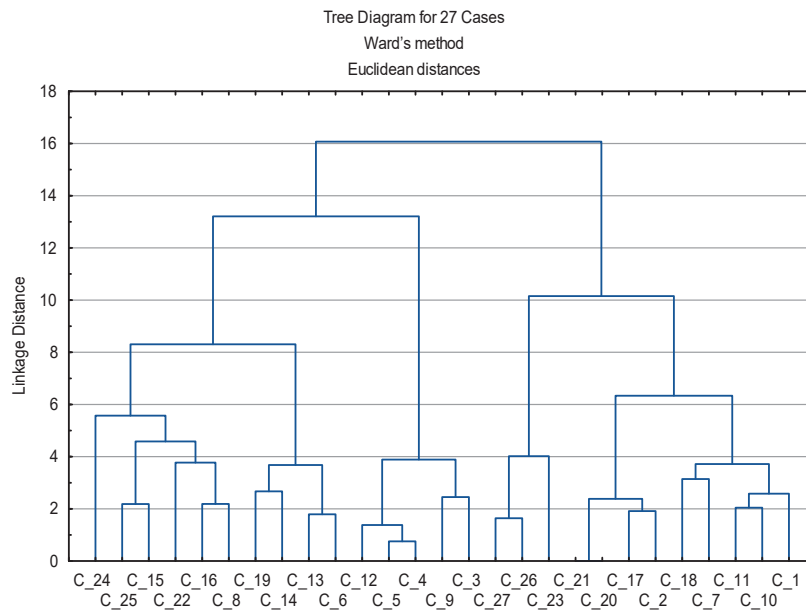


Fig. 6. Hierarchical clustering of patients based on the CD3⁺/CD8⁺ profile (Ward's method, Euclidean distance). Clusters labeled by immune phenotype. The X axis represents individual cases (patients) included in the cluster analysis; the Y axis represents the distance between clusters. The horizontal dashed line corresponds to the dendrogram cut level, at which three immune phenotypes are distinguished: immune-cold, immune-hot, and immune-excluded.

The first cluster included 10 patients (37.04 %) and was characterized by a relatively low density of CD8⁺ cytotoxic T-lymphocytes in the tumor center, with significantly higher values at the invasive margin. The median CD8⁺ cell density in the center was only 1.7 (1.15; 2.08), whereas at the invasive margin, it reached 2.7 (2.38; 3.55). A similar gradient was observed for CD3⁺ cells: 5.7 (4.97; 6.40) in the center versus 12.56 (9.05; 14.05) at the invasive margin. The CD3⁺/CD8⁺ ratio in the tumor center and at the invasive margin was 3.22 and 4.11, respectively.

The second cluster comprised only 5 cases (18.52 %) with the highest values of intratumoral T-cell infiltration among the three clusters. The median density of CD3⁺ cells in the tumor center was 5.10 (4.80; 5.10) and of CD8⁺ cells was 2.70 (2.50; 2.70). T-cell infiltration at the invasive margin was the highest across all clusters: 13.80 (7.30; 14.20) and 6.60 (6.40; 7.30) for CD3⁺ and CD8⁺ lymphocytes, respectively. The CD3⁺/CD8⁺ ratio in the tumor center and at the invasive margin was 1.78 and 2.16, respectively.

The third cluster included 12 cases (44.44 %) with moderate values of CD3⁺ and CD8⁺ cells in both compartments. The median CD3⁺ cell density in the center was 3.31 (2.25; 4.09) and CD8⁺ was 1.43 (0.90; 1.50); at the invasive margin the corresponding values were 5.55 (3.98; 6.63) and 3.25 (2.78; 3.60). The CD3⁺/CD8⁺ ratio in the tumor center and at the invasive margin was 2.35 and 1.93, respectively. Although the median CD8⁺ density at the invasive margin was somewhat higher compared to the immune-excluded phenotype, the overall level of T-cell infiltration remained relatively low.

The dendrogram of the hierarchical cluster analysis using Ward's method (Fig. 6) demonstrates three well-separated groups of patients based on the density of CD3⁺ and CD8⁺ lymphocytes in the tumor center and

at the invasive margin. The distances on the dendrogram reflect the degree of similarity in the immune profile among the patients.

The immune-excluded phenotype was characterized by accumulation of CD3⁺ and CD8⁺ lymphocytes predominantly at the tumor–stroma interface with limited intratumoral penetration. A significant ($p < 0.05$) predominance of CD3⁺ and CD8⁺ T-lymphocyte density at the invasive margin versus the tumor center was identified. Such a phenotype was characterized by the formation of barriers that impede the migration of effector T-cells into the tumor tissue.

The immune-hot phenotype was the smallest cluster, characterized by a high density of T-lymphocytes both in the tumor center and at the invasive margin. Such a phenotype was associated with an active antitumor immune response and high cytotoxic activity of T-cells; however, no statistically significant differences were found in the density of CD3⁺ and CD8⁺ T-lymphocyte between the tumor center and the invasive margin ($p = 0.0625$). The small proportion of this phenotype within the studied cohort indicates that tumors with pronounced intratumoral T-cell infiltration are relatively rare.

The immune-cold phenotype was a cluster characterized by a low-to-moderate density of CD3⁺ and CD8⁺ cells in both tumor compartments; with a significant invasive-margin predominance ($p = 0.00049$). These changes reflected insufficient immune activation and limited involvement of adaptive immunity in controlling tumor growth.

Discussion

Contemporary studies demonstrate that the prognostic significance of T-lymphocyte infiltration in CRC is determined not only by its quantitative characteristics but primarily by

its spatial organization, which dictates the clinical course of the disease [6]. Modern scientific publications emphasize that the spatial evaluation of immune infiltration and the heterogeneity of immune cell distribution between the tumor center and its invasive margin hold independent prognostic value [14].

Furthermore, the density of CD3⁺ and CD8⁺ T-lymphocytes is considered a key characteristic of the antitumor immune response in CRC; assessing their distribution in the tumor center and at the invasive margin provides higher prognostic value than simple confirmation of their presence, owing to pronounced intratumoral heterogeneity and the varying functional roles of immune cells in different tumor zones, as pointed out by several authors [15,16]. Recent studies have established that the total count of CD3⁺ cells correlated with the "stage and the presence of perineural invasion, and a link has also been found between a higher N stage and low counts of CD3⁺ cells [17].

The immunohistochemical results obtained in our study demonstrate that the immune microenvironment of CRC with regional lymph node metastases is characterized by moderate and heterogeneous T-cell infiltration in both the tumor center and at the invasive margin. Along with areas of relatively low T-cell density, zones with more intense infiltration are present, a typical morphological feature of the invasive margin in CRC. In the tumor center, a slightly lower or less organized infiltration was identified, likely related to the formation of an immunosuppressive microenvironment, hypoxia, and impaired access of effector cells to tumor complexes [18].

Analysis of T-lymphocyte subpopulations revealed that the density of CD8⁺ cytotoxic T-lymphocytes was significantly lower compared with CD3⁺ cells, forming a characteristic imbalance: while total T-cell infiltration (CD3⁺) is sufficient, a relative deficit of effector CD8⁺ cells is present. This pattern may indicate predominance of non-cytotoxic subpopulations (CD4⁺ T-helper cells, regulatory T-cells) or reflect functional exhaustion of CD8⁺ lymphocytes. Morphologically, this manifests as the absence of dense, continuous accumulation of effector cells at the tumor – stroma interface. The identified changes allow us to characterize the immune phenotype of the studied tumors as functionally restricted, where infiltration coexists with the functional impairment of T-cells. The literature describes the phenomenon of T-cell exhaustion / dysfunction, which develops under chronic antigenic stimulation and immunosuppressive influence of the tumor microenvironment and is accompanied by increased expression of inhibitory receptors and reduced cytotoxic activity [19].

Cluster analysis confirmed the pronounced heterogeneity of the CRC immune microenvironment. The identification of three phenotypes, immune-hot, immune-cold, and immune-excluded, aligns with contemporary data on tumor phenotypes characterized by distinct spatial T-cell distribution patterns and varying efficacy of the antitumor immune response [20].

The immune-excluded phenotype may be associated with activation of TGF- β -dependent mechanisms of stromal remodeling, increased extracellular matrix density, or other immunosuppressive microenvironmental factors. However, as noted by several authors, the immune-excluded phenotype remains the most poorly defined among the three,

lacks a universally accepted definition, and is frequently associated with poor clinical outcomes [21].

The immune-cold phenotype is characterized by a low density of effector T-lymphocytes, which may be associated with low tumor immunogenicity immunosuppressive microenvironment formation, and insufficient recruitment or functional exhaustion of CD8⁺ T-lymphocytes. This limits effective antitumor immunity and may promote the metastatic progression of CRC [22].

Overall, the predominance of immune-excluded and immune-cold phenotypes (combined 81.48 % of cases) indicates that effective T-cell penetration into the tumor center is limited in the majority of studied tumors with regional lymph node metastasis. Conversely, the 18.52 % of cases presenting as immune-hot tumors suggest an active antitumor immune response; according to literature data, such tumors are associated with a more favorable prognosis and better response to immunotherapy [23]. Meanwhile, it should be taken into account that the development of metastases in regional lymph nodes is a multifactorial process, therefore the spatial organization of the immune microenvironment should be considered as one of the independent potential prognostic factors in combination with other clinical and morphological characteristics of the tumor.

The ratio of immune phenotypes observed in our study is broadly consistent with contemporary concepts regarding the biological heterogeneity of CRC. Given that tumors with a deficient mismatch repair system (MSI-H/dMMR – tumors with high microsatellite instability) are typically characterized by a pronounced infiltration of CD3⁺ and CD8⁺ T-lymphocytes and are considered immune-hot, whereas tumors with a proficient DNA repair system (MSS/pMMR – microsatellite stable tumors with proficient repair) more frequently exhibit an immune-cold or -excluded profile, the identified cluster structure may, hypothetically, reflect the distribution of these molecular subtypes within the studied cohort.

Conclusions

1. In the studied cases of colorectal cancer with metastases to regional lymph nodes, spatial heterogeneity of T-cell infiltration was demonstrated. The density of CD3⁺ and CD8⁺ lymphocytes in all cases at the invasive margin was significantly higher than in the tumor center, indicating a predominance of antitumor immune activity in the tumor-tissue interface zone.

2. The predominance of CD3⁺ cells over CD8⁺ within both studied compartments and the CD3⁺/CD8⁺ ratio indicate the dominance of a non-cytotoxic T-cell population in the microenvironment of colorectal cancer with regional lymph node metastases.

3. Cluster analysis based on CD3⁺ and CD8⁺ infiltration parameters identified three immune phenotypes corresponding to the contemporary immune-hot, immune-cold, and immune-excluded classification. 81.48 % of the studied colorectal cancers with metastases to regional lymph nodes corresponded to the immune-cold and immune-excluded phenotypes, while the immune-hot phenotype, which may be associated with a more pronounced antitumor immune response, was detected in only 18.52 % of cases.

4. The spatial distribution of CD3⁺ and CD8⁺ T-lymphocytes is a crucial characteristic of the colorectal cancer

immune microenvironment. Combined evaluation of these parameters in the tumor center and at the invasive margin enables identification of distinct immune phenotypes and provides a more accurate biological stratification of tumors.

Prospects for future research. Priority directions include a correlation analysis of immune cell density and topography with clinicopathological parameters (TNM stage, differentiation grade, lymphovascular invasion, MSI/MMR status) and long-term treatment outcomes, which will help assess the prognostic value of spatial T-cell infiltration.

Ethical approval

The Bioethics Commission of Zaporizhzhia State Medical and Pharmaceutical University reviewed the materials presented in the article and confirmed that the study complies with applicable ethical standards, including the Declaration of Helsinki, the Council of Europe Convention on Human Rights and Biomedicine, and other relevant legal regulations. Written informed consent was obtained from all participants prior to their inclusion in the study. Ethical approval is documented by an extract from the minutes of the Bioethics Commission meeting (Protocol No. 9, dated June 11, 2026).

AI statement

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