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Prognostic value of tumor-infiltrating T-lymphocytes density in the therapeutic response to initial platinum-based chemotherapy in patients with non-small cell lung cancer

Prognostička vrednost „gustine“ tumor-infiltrišućih T-limfocita u terapijskom odgovoru na inicijalnu hemioterapiju zasnovanu na platini kod bolesnika sa nesitnoćelijskim karcinomom pluća

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Abstract

Background/Aim. The fact that lung carcinomas, like other solid tumors, can be immunogenic may have a substantial prognostic value in non-small cell lung cancer (NSCLC). Specific cytotoxic T-lymphocytes (CTL) can be demonstrated in most patients with primary tumors of different histological types. Two main groups of T-lymphocytes participate in the coupled recognition of tumor-specific antigens – CTL ($CD8^+$) and helper T-lymphocytes ($CD4^+$). The aim of the study was to assess the relationship between the tumor infiltration of T-lymphocytes and the therapeutic response to initial chemotherapy. **Methods.** Data were obtained from patients with NSCLC whose therapeutic response after four cycles of initial platinum-based chemotherapy was observed in relation to the density of tumor-infiltrating T-lymphocytes ($CD4^+$ and $CD8^+$) in small tumor biopsy samples. The therapeutic response was assessed in line with Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 therapeutic response evaluation system. Based on the expected therapeutic response, the patients were divided into three groups: favorable therapeutic response patients (complete and partial regression), stable disease patients, and disease progression patients. To assess the density of $CD4^+$ and $CD8^+$ T-lymphocytes, the number of lymphocytes was determined at ×200 magnification (1.1 mm^2). Three visual fields with the densest lymphocyte infiltrate were selected for counting, and the values of all individual fields were added up. Based on the mean value, the samples were classified into the following groups: score 0, score 1, score 2, and score 3. During statistical data processing, low infiltration density combined score 0 and score 1 groups, and high infiltration density combined score 2 and score 3 groups. Based on the collected data, a database was created in SPSS 22.0 software and used for further statistical analysis. Statistical analysis of the data included descriptive and analytical statistics methods. **Results.** There was no significant difference in the distribution of $CD4^+$ T-lymphocytes and $CD8^+$ T-lymphocytes in the epithelial component of the tumor between patients with a different therapeutic response ($\chi^2 = 2.977$; $p = 0.226$ and $\chi^2 = 1.329$; $p = 0.515$, respectively). There was no significant influence of the infiltration density of $CD4^+$ T-lymphocytes and $CD8^+$ T-lymphocytes in the stromal component of the tumor on the therapeutic response ($\chi^2 = 0.606$; $p = 0.739$ and $\chi^2 = 5.167$; $p = 0.076$, respectively). **Conclusion.** The research did not prove that patients with a high level of tumor-infiltrating $CD4^+$ and $CD8^+$ T-lymphocytes in the epithelial and stromal component of the NSCLC had a better therapeutic response to standard initial chemotherapy.

Key words:
carcinoma, non-small cell lung; cd4-positive t-lymphocytes; prognosis, t-lymphocytes; t-lymphocytes, cytotoxic; treatment outcome.

Apstrakt

Uvod/Cilj. Činjenica da karcinomi pluća, kao i drugi solidni tumori, mogu biti imunogeni, može imati značajnu prognostičku vrednost kod nesitnoćelijskog karcinoma pluća (NSĆKP). Specifične citotoksične T-limfocite (CTL) moguće je dokazati kod većine bolesnika sa primarnim tumorima različitih histoloških tipova. Dve glavne grupe T-limfocita učestvuju u prepoznavanju tumor-specifičnih antigena: CTL ($CD8^+$) i pomoćnički

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(*helper*) T limfociti (CD4+). Cilj rada bio je da se proceni veza između infiltracije T-limfocita u tumor i terapijskog odgovora na inicijalnu hemioterapiju. **Metode.** U istraživanju su korišćeni podaci bolesnika sa NSĆKP, čiji je terapijski odgovor nakon inicijalna četiri ciklusa hemioterapije platinom posmatran u odnosu na „gustinu" tumor-infiltrišućih T-limfocita (CD4+ i CD8+) u malim uzorcima biopsije tumora. Terapijski odgovor procenjivan je u skladu sa *Response Evaluation Criteria in Solid Tumor* (RECIST) 1.1 sistemom procene terapijskog odgovora. Na osnovu očekivanog terapijskog odgovora, bolesnici su bili podeljeni u tri grupe: bolesnici sa povoljnim terapijskim odgovorom (kompletna i delimična regresija), bolesnici u stabilnoj fazi bolesti i bolesnici sa progresijom bolesti. Broj limfocita za procenu gustine CD4+ i CD8+ T-limfocita određen je uz uvećanje x200 (1,1 mm^2). Za brojanje su odabrana tri vidna polja sa „najgušćim" infiltratom limfocita, zatim su vrednosti svih pojedinačnih polja sabrane. Na osnovu srednje vrednosti, uzorci su klasifikovani u sledeće grupe: skor 0, skor 1, skor 2, skor 3. Prilikom statističke obrade podataka, niska gustina infiltracije objedinila je grupe skora 0 i 1, a visoka gustina infiltracije objedinila je grupe skora 2 i 3. Na osnovu prikupljenih podataka kreirana je baza u softveru SPSS 22.0 koja je korišćena za dalju statističku analizu. Statistička analiza podataka obuhvatila je deskriptivne i analitičke statističke metode. **Rezultati.** Nije bilo značajne razlike u distribuciji CD4+ T-limfocita i CD8+ T-limfocita u epitelnoj komponenti tumora između bolesnika sa različitim terapijskim odgovorom (χ^2 = 2,977; p = 0,226 i χ^2 = 1,329; p = 0,515, redom). Nije bilo značajnog uticaja gustine infiltracije CD4+ T-limfocita i CD8+ T-limfocita u stromalnoj komponenti tumora na terapijski odgovor (χ^2 = 0,606; p = 0,739 i χ^2 = 5,167; p = 0,076, redom). **Zaključak.** Istraživanjem nije dokazano da bolesnici sa visokim nivoom tumor infiltririšućih CD4+ i CD8+ T-limfocita u epitelnoj i stromalnoj komponenti NSĆKP imaju bolji terapijski odgovor na standardnu inicijalnu hemioterapiju.

Ključne reči:
pluća, nesitnoćelijski karcinom; limfociti t, cd4; limfociti t; prognoza, limfociti t, citotoksični; lečenje, ishod.

Introduction

The number of patients with lung cancer has been increasing since the late 1960s, and the increase is more pronounced in women [1]. The introduction and application of chemotherapy protocols based on platinum achieved a significant improvement in patient survival. However, a median survival 'plateau', which is still less than one year, has also been reached [2, 3]. Despite the observed progress in treating patients with lung cancer, their survival is still poor. The five-year survival rate of patients with non-small cell lung cancer (NSCLC) is about 60% in those with localized disease, 33% in patients with regionally advanced disease, and only 6% in patients with distant metastases [4, 5]. The high mortality rate from lung cancer is a consequence of its ability to spread locally and/or metastasize very early after its formation. As many as 30–55% of operated stage IB to IIIA patients have recurrence after surgery and die from metastases [6].

The modern therapeutic approach recommends tumor genotyping and identification of mutations in specific oncogenes. Patients with metastatic NSCLC with a targetable oncogenic molecular driver variant and a PD-L1 expression level of 1% or more should receive first-line targeted therapies that yield higher response rates than immune checkpoint inhibitors (lower response rates) [7]. Nevertheless, platinum-based chemotherapy is still seen as the "gold standard" treatment for most patients with NSCLC.

Ideas about a certain connection between immunity and tumors date back to the beginning of the 19th century. Under ideal conditions, the anti-tumor immune response protects the body from developing cancer. This self-perpetuating cyclical process begins with the release of antigen from the tumor cell and ends with the killing of the tumor cell [7]. The key step of this cycle is when T-lymphocytes recognize tumor cells as dangerous. The recognition triggers the effector mechanisms of cellular immunity, i.e., the activation and multiplication of T-lymphocytes entering the bloodstream and reaching the tumor. Based on tumor-presenting antigens, T-lymphocytes attack and destroy cancer cells, thus releasing new antigens that start a new immune cycle [8].

In most patients with primary tumors of different histological types, it is possible to demonstrate specific cytotoxic T-lymphocytes (CTLs). CTLs (CD8+) and helper T-lymphocytes (CD4+) participate in the coupled recognition of tumor-specific antigens. Research shows that abundant infiltration of CTLs indicates a better prognosis and clinical course of the disease [9, 10].

The fact that different types of infiltrating immune cells in lung carcinomas have various effects on tumor progression may have a substantial prognostic value. The literature data support the notion of immune recognition and elimination of malignant cells [11]. The aim of the study was to assess the relationship between the infiltration density of tumor-infiltrating lymphocytes (TIL) and therapeutic response to initial chemotherapy.

Methods

A retrospective/prospective study conducted at the Clinic for Pulmonary Diseases and the Institute of Pathology of the University Clinical Center (UCC) in the Republic of Srpska (RS), Bosnia and Herzegovina, included patients from January 1, 2012, to June 30, 2014. The prospective part of the research lasted until the deadline for the follow-up of the respondents and was completed on June 30, 2017. The study was approved by the Ethics Committee of the UCC, RS (No 01-9-368.2/16 from June 06, 2016).

Patients

The research included 93 patients with NSCLC histologically verified by the Institute of Pathology of the RS UCC. The patients were treated at the Clinic for Pulmonary Diseases of the RS UCC.

Study design

To include patients in the research, the following was required: a histological diagnosis of NSCLC; the absence of previously diagnosed cancer of the lungs or other organs; the patients had to be radio-/chemonaive; locally advanced or metastatic disease [the tumor-T, node-N, and metastasis-M (TNM) – IIIA, IIIB, IIIC or IVA and IVB stage] confirmed clinically; the absence of epidermal growth factor receptor mutations.

Based on the expected therapeutic response, the patients were divided into three groups: favorable response patients (complete and partial regression), stable disease patients, and disease progression patients. The therapeutic response was assessed after the initial four cycles of platinum chemotherapy, in line with Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 therapeutic response evaluation system: Stable Disease; Partial Response – 30% size reduction of the target lesion taking the initial dimension of the target lesion as a reference; Complete Response – complete disappearance of extranodal lesions with reduction of nodal lesions to < 10 mm in shorter diameter and disease progression; Progressive Disease – minimum 20% lesion size increase.

Evaluation of $CD4^+$ and $CD8^+$ T-lymphocytes density was accomplished with samples obtained by tumor biopsy before initial chemotherapy. For histological and immunohistochemical (IHC) analysis, the formalin-fixed and paraffin-embedded tissues were cut into 4–5 μm thick semiserial sections on a rotary microtome (microTec Cut 4055 SLEE MAINZ). After staining, according to the standard protocol for the routine hematoxylin-eosin method, the tissues are mounted in dibutylphthalate polystyrene xylene medium and analyzed with an Olympus BX41 light microscope (Olympus, Tokyo, Japan). For correct histological differentiation of lung tumors, IHC staining was used with antibodies for CK7, p63, TTF1, CK5/6, napsin A, synaptophysin, and chromogranin A. The prepared tumor tissues were IHC analyzed according to the selected antibodies range on an automated platform for IHC staining (Ventana BenchMark GX). All used monoclonal antibodies (SP35, SP57, SP141, SP52, D5/16B4, 4A4, SP11, LK2H10, MRQ-60) for immunophenotyping of tumor cells and detection of $CD4^+$ and $CD8^+$ T-lymphocytes were optimally diluted (ready-to-use format) and compatible with the detection system for visualization (ultraView Universal DAB detection kit) and other Ventana Medical Systems, Inc. reagents prepared for automatic staining. The software-designed protocol for antigen visualization included tissue deparaffinization, antigen unmasking, visualization of immune deposits in the tissue, and contrasting the surrounding tissue. Verified positive and negative control tissue was added to each tumor tissue sample to control the quality of IHC staining. The number of $CD4^+$ and $CD8^+$ T lymphocytes with positive staining were counted in epithelial and stromal components of the tumor (Figures 1 and 2). To assess the density of $CD4^+$ and $CD8^+$ T-lymphocytes, the number of lymphocytes was determined at ×200 magnification (1.1 mm^2). Three visual fields with the densest infiltrate were selected for counting, and the values of all individual fields were added up. The preparations were read by a pathologist using a four-tiered scale based on the mean value: score 0 (no lymphocytes or without inflammatory infiltration), score 1 (1–19 lymphocytes or rare infiltration densi-

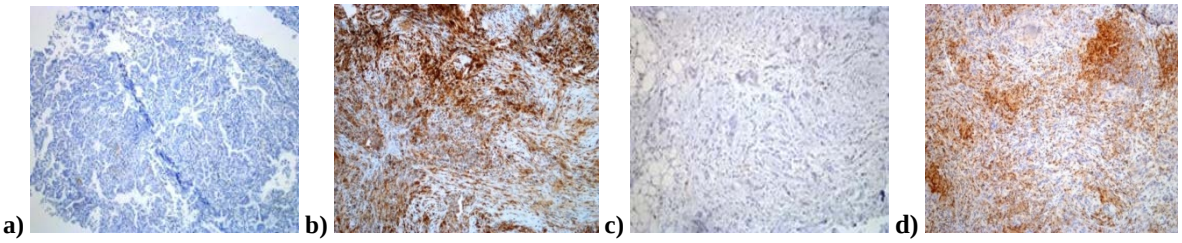

Fig. 1 – Immunohistochemical staining of tumor tissue with anti-CD4 antibody: a) Low density of $CD4^+$ T-lymphocytes in the epithelial component; b) High density of $CD4^+$ T-lymphocytes in the epithelial component; c) Low density of $CD4^+$ T-lymphocytes in the stromal component; d) High density of $CD4^+$ T-lymphocytes in the stromal component (×200 magnification, 1.1 mm^2).

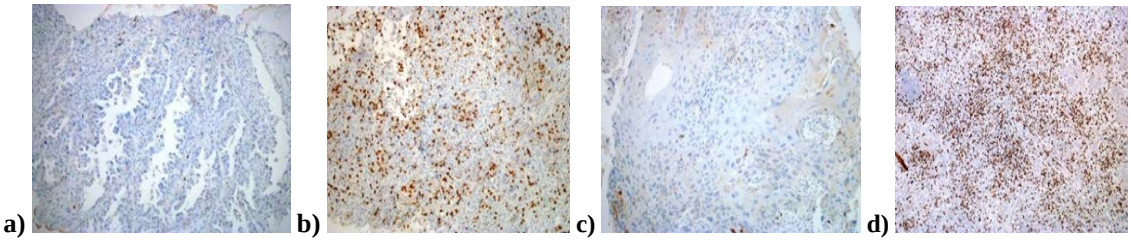

Fig. 2 – Immunohistochemical staining of tumor tissue with anti-CD8 antibody: a) Low density of $CD8^+$ T-lymphocytes in the epithelial component; b) High density of $CD8^+$ T-lymphocytes in the epithelial component; c) Low density of $CD8^+$ T-lymphocytes in the stromal component; d) High density of $CD8^+$ T-lymphocytes in the stromal component (×200 magnification, 1.1 mm^2).

ty), score 2 (20–49 lymphocytes or moderately infiltration density), and score 3 (more than 50 lymphocytes or high infiltration density) [12, 13]. During statistical processing of the data, due to the small number of samples in the group scores 2 and 3, case patients with TIL categories 0 to 1 were put in the low-TIL group, and those with scores 2 and 3 were grouped as high-TIL tumor patients.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Version 22; SPSS, Chicago, IL, USA). Descriptive data for all groups and variables will be presented as numbers and percentages and compared with the χ^2 test. The independent variable used in the research is a therapeutic response. A statistically significant difference was defined at the $p < 0.05$ level and a difference of very high statistical significance at the $p < 0.01$ level.

Results

The obtained data showed no patient with a complete therapeutic response.

We did not prove any differences in the distribution of $CD4^+$ T-lymphocytes in the epithelial component of the tumor between patients with a different therapeutic response ($\chi^2 = 2.977$; $p = 0.226$). Moreover, we did not prove any significant differences in the distribution of $CD8^+$ T-lymphocytes in the epithelial component of the tumor between patients with a different therapeutic response ($\chi^2 = 1.329$; $p = 0.515$) (Table 1, Figure 3).

There was no statistically significant influence of the infiltration density of $CD4^+$ T-lymphocytes in the stromal component of the tumor on the therapeutic response ($\chi^2 = 0.606$; $p = 0.739$). Also, there was no statistically significant influence of the infiltration density of $CD8^+$ T-lymphocytes in the stromal component of the tumor on the therapeutic response ($\chi^2 = 5.167$; $p = 0.076$) (Table 2, Figure 4).

Table 1

Distribution of patients in relation to the density of infiltration $CD4^+$ and $CD8^+$ T-lymphocytes in the epithelial component of the tumor

Response to therapy	Low density		High density	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Partial response	27 (38.0)	24 (36.9)	4 (18.2)	7 (25)
Stable disease	22 (31.0)	21 (32.3)	9 (40.9)	10 (35.7)
Progressive disease	22 (31.0)	20 (30.8)	9 (40.9)	11 (39.3)
Total	71 (100.0)	65 (100.0)	22 (100.0)	28 (100.0)

All values are expressed as numbers (percentages).

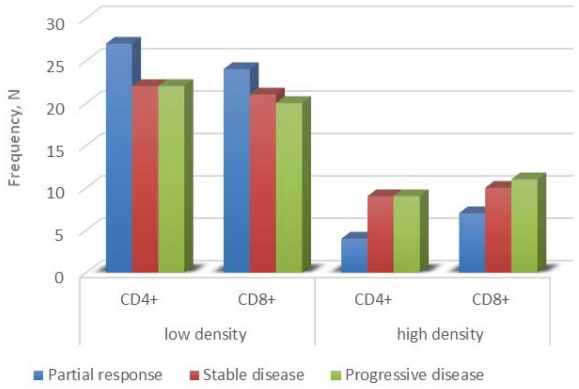

Fig. 3 – Distribution of patients in relation to the density of infiltration of $CD4^+$ and $CD8^+$ T-lymphocytes in the epithelial component of the tumor.
N – number.

Table 2

Distribution of patients in relation to the density of infiltration CD4+ and CD8+ T-lymphocites in the stromal component

Response to therapy	Low density		High density	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Partial response	14 (32.6)	14 (38.9)	17 (34.0)	17 (29.8)
Stable disease	13 (30.2)	7 (19.4)	18 (36.0)	24 (42.1)
Progressive disease	16 (37.2)	15 (41.7)	15 (30.0)	16 (28.1)
Total	43 (100.0)	36 (100.0)	50 (100.0)	57 (100.0)

All values are expressed as number (percentages).

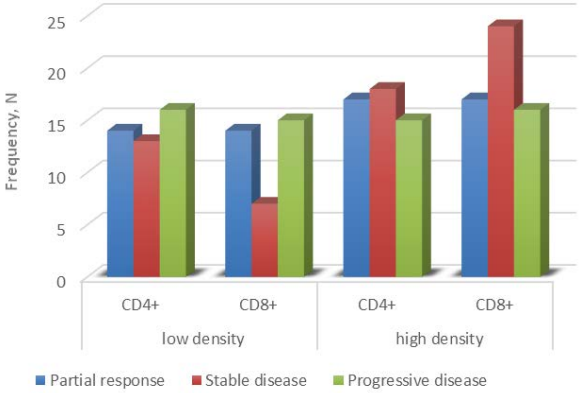

Fig. 4 – Distribution of patients in relation to the density of infiltration of CD4⁺ and CD8⁺ T-lymphocytes in the stromal component of the tumor.
N – number.

We did not prove that the density (low/high) of the inflammatory CD4⁺ T-lymphocyte infiltrate in the epithelial component of the tumor [χ^2 (1) = 2.977; p = 0.084] and the stromal component of the tumor [χ^2 (1) = 0.022; p = 0.883] were relevant for partial response to initial chemotherapy.

The results of the Pearson's χ^2 test [χ^2 (1) = 5.099; p = 0.024] demonstrate a significant difference in the distribution of patients in terms of response to therapy (stable disease) based on CD8⁺ T-lymphocytes density in the stromal component of the tumor (Table 2, Figure 4).

Discussion

Despite evident progress and the discovery of new molecules (target therapy and immunotherapy), conventional platinum-based chemotherapy remains the "gold standard" for most patients suffering from NSCLC. Initial treatment involves four cycles of chemotherapy. In addition to platinum, the most frequently added are etoposide, gemcitabine, paclitaxel, docetaxel, or vinorelbine because combined therapies provide a better therapeutic response and overall survival (OS) rate compared to those without platinum. Chemotherapy with cisplatin results in a small but statistically significant improvement in survival compared with good supportive care [14, 15]. The median OS in patients with advanced NSCLC not treated with chemotherapy is 4.5 months. The use of chemotherapy improves the one-year OS rate from 10–20% to 30–50% [16].

The clear superiority of one platinum-containing combination over another was demonstrated in controlled trials [17]. The most frequently applied protocols in our research were cisplatin-etoposide, 44 (47.3%), and cisplatin-gemcitabine, 28 (30.1%). Other platinum-based protocols used included paclitaxel, docetaxel, and vinorelbine.

There are two components of tumor tissue – parenchyma (made up of tumor cells) and tumor stroma. These two components are closely related. Parenchyma is the part of the tumor that determines its biological properties. Tumor stroma consists of an extracellular matrix, blood vessels, and cellular elements that define tumor growth and evolution. Inflammatory cells also belong to the cellular components of the tumor stroma. T-lymphocytes are a significant factor for local (*in situ*) tumor immunity. Conducted studies on the prognostic role of immune cells in NSCLC gave contradictory results [18–20]. In searching for an answer to the connection between a high density of lymphocyte infiltration and therapeutic response, it is necessary to distinguish subtypes of TIL [21].

Regarding the research results, we concluded that the density (low/high) of inflammatory CD4⁺ T-lymphocyte infiltrate in the epithelial and stromal components was irrelevant for optimal response to the initial chemotherapy.

A systematic review and meta-analysis of studies that gave a correlation between the infiltration density of CD4⁺ T-lymphocytes and OS and progression-free survival showed that CD4⁺ T-lymphocytes were associated with slightly improved OS (hazard ratio: 0.82; 95% confidence interval: 0.69–0.98) [22]. A positive relationship between tumor-infiltrating CD4⁺ T-lymphocytes density and the response to chemotherapy in an examination of the density of TIL (high and low) and the response to chemotherapy was not observed by pooling patients with complete and partial responses [23]. CD4⁺ T-lymphocytes are thought to have the ability to suppress or regulate cellular immunity. However, their presence can potentially promote tumor growth. That indicates their inability to provide an effective immune response and leads to the conclusion that tumor-infiltrating T-lymphocytes are functionally impaired and incompletely activated and that they include regulatory subtypes that vary depending on the type of cancer [24].

Research performed to clarify the relationship between the number of tumor-infiltrating T-lymphocytes and clinical and pathological characteristics and clinical outcomes in patients with NSCLC showed that individual high density of CD4⁺ or CD8⁺ T-lymphocytes had no prognostic significance for therapeutic response to conventional chemotherapy. Although the level of infiltration by CD8⁺ T cells alone had no prognostic significance, the survival rate was signifi-

cantly higher for patients with both 'high' $CD8^+$ and 'high' $CD4^+$ T-cell infiltration than for the other two groups according to whether their tumors exhibited a 'high' or 'low' level of $CD4^+$ or $CD8^+$ T-lymphocyte infiltration (log-rank test, $p = 0.006$) [25].

Follow-up of the OS rate did not identify an interaction between TIL and chemotherapy treatment, as the OS rate in patients who received and those who did not receive chemotherapy was 0.88 and 0.90, respectively [26].

The research did not find any significant differences in the frequency distribution of $CD8^+$ T-lymphocytes in the epithelial and stromal components in patients with a favorable therapeutic response. The level of inflammatory $CD8^+$ T-lymphocyte infiltrate in the stromal component of the tumor had positive prognostic significance in patients with stable disease.

Literature data concluded that a high $CD8^+$ T-lymphocyte infiltration level in NSCLC is a favorable prognostic factor [23, 27]. Assessing $CD8^+$ T-lymphocyte infiltration in the stromal component of the tumor is more significant for prognostic value than assessing $CD8^+$ T-lymphocyte infiltration in the epithelial component of the tumor [28]. Although estimating the numbers of macrophages and $CD8^+$ T-cells in cancer nests and stroma are valuable biomarkers for predicting the prognosis of stage IV NSCLC patients treated with chemotherapy, we still cannot forecast the response to chemotherapy by counting them [29].

Meanwhile, numerous available research results did not prove any prognostic significance for total intraepithelial T cells and their subtypes ($CD8^+$ and $CD4^+$ T-lymphocytes) in patients with NSCLC [30].

The ability of tumor cells to produce immunosuppressive factors in the microenvironment, thereby impairing the ability of $CD8^+$ T-lymphocytes-mediated tumor cell lysis, might be a possible explanation for the results of this research [31, 32]. Most tumor antigens recognized by $CD8^+$ T-lymphocytes are mainly endogenously synthesized cytoplasmic or nuclear proteins displayed in complex with major histocompatibility complex (MHC) class I molecules. Low cytotoxicity of $CD8^+$ T-lymphocytes is caused by poor expression of the MHC class I complex performed by tumor cells [33]. $CD8^+$ T-lymphocytes can only recognize antigens within MHC class I molecules. That is also because the proliferation and differentiation of $CD8^+$ T cells in CTL require co-stimulation and/or the help of $CD4^+$ T-lymphocytes, which recognize peptides in complex with MHC class II molecules. TIL density assessed in small biopsy samples is valid. However, it cannot reliably represent their density in the entire tumor because of the heterogeneity of $CD8^+$ T-lymphocytes in NSCLC. That is why additional studies are recommended before valid clinical conclusions can be drawn. These studies would directly measure the prognostic or predictive value of $CD8^+$ T-lymphocyte count in small biopsy specimens [34].

Limitations of the study

The shortcomings of the conducted research are the sample size. We should assume that the observed occurrences regarding the sample size would also gain statistical significance. Other shortcomings of the conducted research are the small bioptic samples.

Conclusion

The prognostic significance of TIL is controversial. Different tumor sampling strategies can give inconsistent results on the density of TIL and change the identification of tumors with a high density of TIL, which requires the standardization of IHC staining, scoring system, and localization. The spatial distribution of tumor-infiltrating cells in the tumor microenvironment is heterogeneous, so counting T-lymphocytes in small biopsy samples cannot reliably represent their density in the entire tumor. Future research should focus on the functional analysis of $CD4^+$ and $CD8^+$ T-lymphocytes and assess the inflammatory infiltrate density as a prognostic factor in immunotherapy.

Conflict of interest

The authors declare no conflict of interest.

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