*Article received on September 29, 2017 and accepted for publishing on November 14, 2017.*

# Study of the type and distribution of tumor infiltrating lymphocytes in 27 cases of lung adenocarcinoma

**Andreea Mihai[2], George Simion[1], Chirculescu Florin[1] , Maria Sajin[1,2]**

**Abstract**: *Pathologists observed the presence of tumor infiltrating lymphocytes (TILs) in solid tumors many years before the research focus shifted from cancer cells to the tumoral microenvironment. In lung adenocarcinoma TILs vary in number, phenotype and distribution. Our study investigate the type and distribution of TILs regarding the stage of disease and histological subtype in 27 cases of lung adenocarcinoma. By immunohistochemistry we evaluated semi-quantitatively CD3, CD8 and cyclin D1. We have found that CD3+CD8- lymphocytes prevail at the invasive margin and in tumoral stroma. Cyclin D1 was diffusely positive in tumoral cells in all the five cases of lepidic type of lung adenocarcinoma. In other histological types cyclin D1 was focally positive, most at the margin of the tumor irrespective of the presence or absence of TILs.*

**Keywords**: *lung adenocarcinoma, tumor infiltrating lymphocytes (TILs), CD3, CD8 and cyclin D1*

## INTRODUCTION

In solid tumors, tumoral stroma acts as an interface between the body and cancer and its effectors are fibroblasts and inflammatory cells. Inflammatory cells are mainly lymphocytes (tumor infiltrating lymphocytes/TILs) and few macrophages, dendritic cells and neutrophils. In line with step-wise modulated activity of immune cells, it is a deductive reason that tumor infiltrating lymphocytes have impaired activity caused by peculiar biochemical condition in tumoral microenvironment. Types of lymphocytes are defined by expression of markers that underlie immune competence or suppression, such as CD3, CD8, Foxp3, PD1 and CTLA4. Lymphocytes have a variable presence in lung adenocarcinomas and they vary considerably in number, phenotype and function [1,2,3].

The prognostic value of lymphocytes in cancers demonstrate an organ restriction efficiency of TILs, in few tumors it was shown that presence of TILs has positive impact on evolution, as in melanoma, colon cancer with micro-satellite instability or renal cancer [6,7,8]. Increased level of TILs has been positive correlated with survival [2] and it was shown to be an independent prognostic marker for lung adenocarcinoma patients [3].

Topographical distribution of lymphocytes and their phenotype suggest different triggers for intratumoral lymphocytes and peritumoral lymphocytes at the invasive front. The molecular structure of the "tumoral

[1] Emergency University Hospital Bucharest

[2] Pathology Department, Faculty of Medicine, Carol Davila University of Medicine and Pharmacy, Bucharest

Corresponding author: Andreea Mihai MD
adya_ag@yahoo.com

antigen" is still elusive, while the dynamic balance of simultaneous activator and inhibitory signals in the tumoral microenvironment interfere with non-self-recognition capacity of the lymphocytes.

## MATERIAL AND METHOD

In this study we investigated by immunehisto-chemistry the type and distribution of lymphocytes that infiltrate tumoral stroma and peritumoral areas in 27 cases of lung adenocarcinomas. These cases were selected from 43 lung adenocarcinomas that have been diagnosed and operated in Emergency University Hospital Bucharest between January 2013 and June 2016. CD3 and CD8 expression of tumor infiltrating lymphocytes were evaluated by immunohisto-chemistry on paraffin-embedded tissue.

Additionally, we investigated the cyclin-D1 mediated DNA synthesis in tumoral cells looking for patterns of positivity by immunohistochemistry for cyclin-D1 in relation the presence and density of lymphocytes in vicinity and homogeneity versus clusters of positivity.

**Table 1**

| | Age | Gender | Stage of disease | Histological type | Grade of differentiation | Follow-up period* (*months*) |
|---|---|---|---|---|---|---|
| 1 | 43 | M | IV | mixt | G1 | 19 |
| 2 | 57 | M | IA | lepidic | G3 | 73 |
| 3 | 47 | M | IIA | lepidic | G3 | 16 |
| 4 | 81 | M | IA | lepidic | G3 | 4 |
| 5 | 61 | M | IIB | mixt | G1 | 13 |
| 6 | 67 | M | I A | mixt | G2 | 22 |
| 7 | 48 | M | IIB | mixt | G3 | 34 |
| 8 | 61 | M | IA | mixt | G3 | 1 |
| 9 | 62 | M | III | mixt | G2 | 36 |
| 10 | 59 | F | IB | mixt | G2 | 3 |
| 11 | 75 | F | IA | mixt | G2 | 1 |
| 12 | 65 | M | IIIB | mixt | G3 | 13 |
| 13 | 64 | M | - | lepidic | G2 | 18 |
| 14 | 64 | M | IB | acinar | G1 | 22 |
| 15 | 56 | M | IA | mixt | G1 | 1 |
| 16 | 62 | M | - | mixt | G3 | 1 |
| 17 | 67 | M | IA | lepidic | G3 | 18 |
| 18 | 57 | M | IB | mixt | G2 | 2 |
| 19 | 62 | F | IIB | mixt | G2 | 7 |
| 20 | 46 | M | IA | mixt | G2 | 9 |
| 21 | 62 | M | IB | acinar | G2 | 10 |
| 22 | 70 | F | IA | acinar | G1 | 12 |
| 23 | 59 | F | IA | mixt | G2 | 3 |
| 24 | 70 | M | IV | acinar | G2 | 1 |
| 25 | 56 | M | I | acinar | G2 | 1 |
| 26 | 68 | M | IIA | acinar | G2 | 1 |
| 27 | 70 | M | IV | acinar | G2 | 6 |

**Follow-up period was measured as the interval from diagnosis and the last medical record for the patient in the hospital database*.

Age, gender, stage of disease, histological type of tumor, grade of differentiation (G) and follow-up period from hospital records were taken into account.

The selection of cases for immunehisto-chemistry investigation was made by evaluating hematoxylin-eosin slides of each case for the presence of tumor infiltrating lymphocytes. Positive criteria were 20 lymphocytes at the invasive front or in tumoral stroma or between tumoral cells present at least on 6 high power fields (HPF). Cases with massive necrosis were excluded, although some of these showed limited areas of abundant infiltrating lymphocytes.

In Table 1 details of the each patient are shown.

The mean age of diagnosis was 61 years, between 43 and 81. 81.5% were male and 18.5% were female. The mean follow-up period was 13 months.

## RESULTS

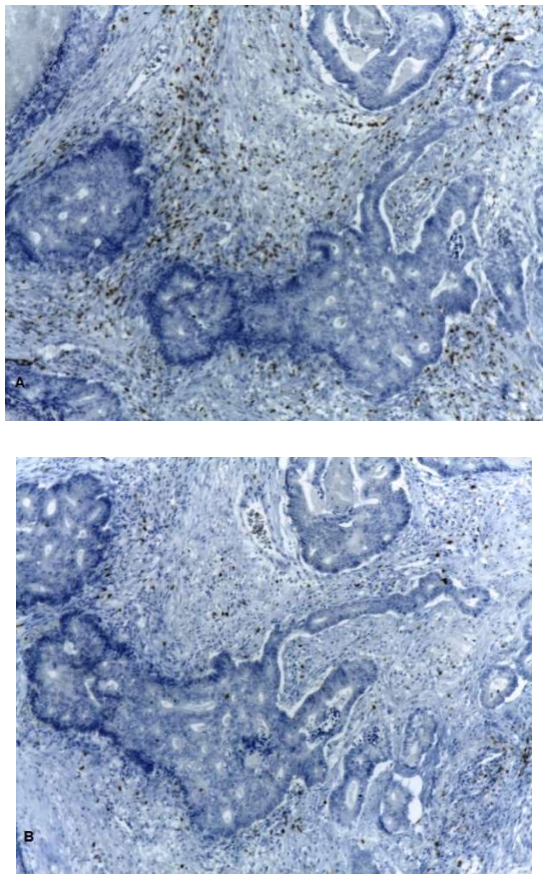
Examination of the hematoxylin-eosin slides showed a topographic variation of the constitutive elements. In the center of the tumor prevails desmoplastic reaction and/or

areas of necrosis, while tumoral cells form small groups and collapsed tubes surrounded by cancer associated fibroblasts (CAFs), collagen fibers and rare inflammatory cells. Necrosis rarely appear at less than 0.5 cm from the margin of the tumor and it was absent in lepidic type of lung adenocarcinoma as we found in our five cases of this type. Necrosis appear at the margin of tumor in the invasive front in cases diagnosed in advanced stages, III and IV.

**Table 2**

| | Histological type | | | Grade of differentiation | | | Stage of disease | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | acinar | mixt | lepidic | G1 | G2 | G3 | I | IIA | IIB | IIIA | IIIB | IV |
| N (%) | 7 (26) | 15 (55) | 5 (19) | 5 (19) | 14 (52) | 8 (29) | 15 (55) | 2 (7) | 3 (11) | 1 (4) | 1 (4) | 3 (11) |

**Figure 1A**. CD3 positive in most of the TILs. 20X. B. Same area, CD8 positive in rare TILs; 20x.

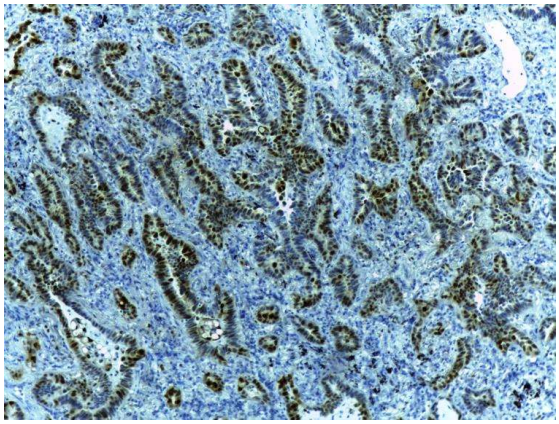



We identified three patterns of morphologic distribution of tumor infiltrating lymphocytes (TILs) as: intratumoral TILs – when infiltrate among tumoral cells (in proximity with them), stromal TILs – when infiltrate in connective stroma between acini and groups of tumoral cells, and peritumoral lymphocytes – when surround discrete or continuously the tumor at the invasive front. Two or all three of these patterns coexist in each tumor.

An inconstant finding was the presence of tertiary lymphoid structures in normal tissue in vicinity of tumor at 0.5-1 cm.

Depending on histological subtype of lung adeno-carcinoma, lepidic type showed the following peculiar aspects: two out of five cases showed homogeneous moderate nuclear positivity for cyclin D1 in>80% of tumor cells irrespective of distance from the margin of the tumor, necrosis was absent and lymphocytes were found to have scarce stromal and discrete peritumoral distribution, and they were predominantly CD3+/CD8-.Stromal TILs were discontinuous, from absent to 50 lymphocytes on HPF, peritumoral TILs were discrete, intratumoral TILs were absent.

**Figure 2.** Cyclin D1 diffuse positive in lepidic lung adenocarcinoma; 10x.



The other types of lung adenocarcinoma included in this study, acinar and mixt, (the mixt type was

represented by predominantly acinar plus other pattern, solid type being identified most of the time), showed important areas of necrosis and desmoplastic reaction in the center of the tumor.

When necrosis was near the margin, it was also found unexpected presence of intratumoral TILs dispersed among tumoral cells with immunophenotype CD3+/CD8+ and increased peritumoral TILs.

Regarding the stage of disease, we have found that in the later stages of disease (stage III and IV) peritumoral lymphocytes are increased, in continuous distribution, with no significant variation regarding stromal or intratumoral lymphocytes, and that CD3+CD8-/ CD3+CD8- ratio always favor CD3+CD8-.

We interpreted that cyclin-D1 showed a gland cluster distribution when positive signals are present in most of the cells in the same gland and we suppose that those cells are in the same developmental stage.

Positivity of cyclin D1 at the margin of tumor is increased compared to the center of the tumor, irrespective to the presence or absence of peritumoral lymphocytes.

TILs in lung adenocarcinoma are predominant CD3+CD8-, they are frequently found at the invasive front, they are variable present in desmoplastic stroma and scarce to absent among tumoral cells.

## DISCUSSIONS

Because of their potential cytotoxic ability, TILs are regarded as promising anticancer therapeutic targets. PD1 and CTLA-4 are the former immune-checkpoint with inhibiting effect on T-cell after their ligands binding [11]; in clinical practice, monoclonal antibodies with PD1 and CTLA-4 blocking activity show inconstant efficiency.

The variable presence of TILs could be due to variable immunogenity of tumor, due to the process of positive and negative clonal selection of lymphocyte during their maturation in lymphoid organs when tolerance cover a variable spectrum of “self” and “non-self” molecules that may be native or altered proteins, which produces variation among individuals and also resistance to cancer, or due to contradictory signals in tumor microenvironment, both proinfla-mmatory and inhibitory cytokines.

The variable distribution of TILs, mainly in stroma and peritumoral, and rarely in direct contact with tumoral cells, make the stroma an agent of sequestration for immune cells, or, from the other side, an agent of compartmentalization of tumoral cells in order to escape the immune system. In tumoral stroma, the inhibiting effect on TILs is orquestrated by CAFs (cancer associated fibroblasts) which secrete soluble cytokines which interfere with all the immune cells in the milieu.

Anterior studies predicted CD4+/CD8- phenotype predominance of TILs [1, 2]. We have sustaining results regarding CD3+ and CD8- lymphocytes. Confirmation of the presence of TILs is the first step in promoting immune-checkpoints targeted therapy. The actual role of the pathologist may be to attest the presence of TILs and to quantify the signal of the target molecule (PD1/PD-L1) in tumoral tissue.

As known, cyclin D1 has a role in promoting cell-cycle progression from G1 to S phase, when the DNA duplicate. In cancers cyclin D1 may be a driving factor (as in mantle cell lymphoma) and sometimes just a secondary event. Cyclin D1 was shown to have role also in genome stability [12].

The cluster positivity of cyclin D1 could suggest that cells derived from the same progenitor have a cell-cycle coherence; we could speculate that this is maintained until disproportionate access to nutrients favor clonal selection for expansion. The lack of correlation between TILs and tumoral cells expressing cyclin D1 apparently exclude a mitogenic effect of infiltrating lymphocytes on cancer cells as we might presume if TILs appear to express more inflammatory then cytotoxic qualities.

## CONCLUSIONS

TILs are potential allies in anti-cancer therapeutic strategy. CD8 positive TILs predict a better outcome in lung adenocarcinoma.

Determinants of the variable presence and topographical distribution of TILs have yet to be

explained.

Cyclin D1 is variably express among histological types of lung adenocarcinoma, with increased expression in lepidic type, and is not correlated with the presence or absence of TILs.

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