ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Tumor microenvironment in squamous cell lung cancer – histological and immunohistochemical study

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Abstract

Globally, lung cancer is the primary cause of cancer-related mortality. Squamous cell carcinoma (SCC) of the lung, a subtype of non-small cell lung cancer (NSCLC), accounts for a significant portion of new cases and is primarily associated with smoking and environmental pollutants. This study investigates the histopathological and immunohistochemical (IHC) characteristics of the tumor microenvironment (TME) in SCC using tissue samples from 19 patients who underwent surgery for lung cancer. IHC markers, including cluster of differentiation (CD)68, CD3 and CD34 were used to assess various components of the TME, including immune cell infiltration, and angiogenesis. The results revealed significant presence of macrophages, T-lymphocytes, and myofibroblasts, as well as increased vascularization in the tumor stroma. These findings highlight the complex interaction between tumor cells and the surrounding stroma, contributing to tumor progression. Understanding these mechanisms is crucial for improving early diagnosis and therapeutic strategies for SCC.

Keywords: squamous cell carcinoma, tumor microenvironment, inflammatory cells, angiogenesis.

Introduction

Lung cancer is characterized by a significantly increased mortality rate in both less developed countries and socio-economically and educationally advanced states [1]. According to some studies, every year, about two million new cases of lung cancer are reported around the world, and 1.76 million people die from this pathology [2, 3]. Lung cancer was the most frequently diagnosed cancer in 2022; it determined approximately 2.5 million new cases, representing one in eight cancers worldwide (12.4% of all cancers worldwide) [4, 5]. The most common subtype of lung cancer, non-small cell lung cancer (NSCLC), which includes the subtypes of lung squamous cell carcinoma (SCC) and lung adenocarcinoma, accounts for 85% of newly diagnosed cases each year. Among the risk factors, active or passive exposure to tobacco smoke is undoubtedly a key factor in the development of NSCLC [6, 7]. NSCLC is most often a very malignant tumor, usually diagnosed in advanced stages [8]. SCC of the lung originates from the basal cells of the pulmonary airways and is characterized by keratinized regions and intercellular bridges [9, 10]. The main cause of the high mortality rate is the late diagnosis of lung pathology [1, 11]. Establishing a definitive diagnosis in the early stages of the disease is crucial for achieving optimal therapeutic outcomes [1]. Early detection of lung cancer can be achieved through the implementation of large-scale prevention programs targeting populations at moderate or high risk [1]. People predisposed to developing this type of cancer include those who work in toxic environments or in areas with excessive pollution, as well as smokers [1]. Thus, we emphasize the importance of the epigenetic component of this pathology [12]. The treatment of lung cancer has advanced significantly compared to

the last decade. Currently, treatment focuses on targeted molecular therapies that are specific and ensure a prompt response and immunotherapy. Molecular therapies for lung cancer predominantly target: the epidermal growth factor receptor (EGFR), epithelial–mesenchymal transition, and fibroblast growth factor receptor [13].

Aim

The aim of this study was to evaluate the histopathological (HP) and immunohistochemical (IHC) characteristics of the tumor microenvironment (TME) in SCC of the lung.

Materials and Methods

In this study, we analyzed lung tissue fragments from 19 patients who underwent surgery for lung cancer at the Thoracic Surgery Clinic of the Emergency County Hospital in Craiova, Romania, fragments that upon HP examination proved to be SCC of the lung. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova (UMPhCv), under number: 73/07.09.2020. The study complies with the requirements of the Ethics Committee of the UMPhCv and was conducted in accordance with national and international legislation, as well as all General Data Protection Regulations (GDPRs) in force.

Histopathological analysis

Fragments of lung tumors were fixed immediately after excision in a 10% neutral buffered formalin solution for 72 hours. These were then embedded in paraffin using the classical histological paraffin embedding procedure. Paraffin blocks were sectioned at the Microm HM325 rotary microtome, obtaining 5 μm thick sections which were placed on simple histological slides and slides treated with poly-L-lysine and stained with HE.

Special techniques were used for IHC studies. Thus, the slides were deparaffinized with xylene in three 15-minute washes, rehydrated with alcohol in decreasing concentrations of 100%, 90%, and 70% (three minutes each solution), and finally washed with distilled water (dH_2O) in three baths of five minutes each. Antigen retrieval was performed by boiling in a microwave oven in ethylene-diaminetetraacetic acid (EDTA) pH 9 or citrate pH 6 solution (according to each antibody's protocol), followed by blocking endogenous tissue peroxidase with 3% hydrogen peroxide (30 minutes). To block endogenous immunoglobulins from the biological material, the slides were introduced into a 3% powdered milk solution in phosphate-buffered saline (PBS), after which the primary antibody (Table 1) was applied for 13 hours at 4°C. The next day, the slides were left at room temperature for 30 minutes, after which they were washed well with PBS to remove excess primary antibody. The secondary antibody [mouse/rabbit immunoglobulin G (IgG), VC002-025, R&D Systems, VisUCyte polymer with horseradish peroxidase (HRP)] was applied to the slides with the biological material for one hour. Excess secondary antibody was removed by washing with PBS for five minutes, after this the 3,3'-Diaminobenzidine (DAB) (Dako) chromogen was applied to the slides under microscopic control, and counterstained (nuclei) with Hematoxylin solution. The slides were dehydrated in alcohol solutions (70%, 90%, and 100% for five minutes each), clarified in xylene for 3×15 minutes. Finally, the slides were mounted using Canada balsam.

Table 1 – ***Immunohistochemical antibody panel***

Antibody	Manufacturer	Clone	Antigenic exposure	Secondary antibody	Dilution	Labeling
Anti-CK7	Dako	OV-TL 12/30	Citrate, pH 6	Monoclonal mouse anti-human CK7	1:50	CK7
Anti-p53	Dako	DO-7	EDTA, pH 9	Monoclonal mouse anti-human p53 protein	1:50	Nuclear marker
Anti-α-SMA	Dako	1A4	Citrate, pH 6	Mouse anti-human SMA	1:100	Myofibroblast cells
Anti-CLA	Dako	2B11+PD7/26	Citrate, pH 6	Monoclonal mouse anti-human CD45	1:50	Lymphocytes
Anti-CD68	Dako	KP1	Citrate, pH 6	Monoclonal mouse anti-human CD68	1:100	Macrophages
Anti-CD34	Dako	QBEnd/10	Citrate, pH 6	Monoclonal mouse anti-human CD34 Class II	1:50	Blood vessels
Anti-CD3	Dako	Polyclonal	Citrate, pH 6	Polyclonal rabbit anti-human CD3	1:50	T-lymphocytes
Anti-CD20	Dako	L26	Citrate, pH 6	Monoclonal mouse anti-human CD20cy	1:50	B-lymphocytes

α-SMA: Alpha-smooth muscle actin; CD: Cluster of differentiation; CK7: Cytokeratin 7; CLA: Cutaneous lymphocyte antigen; EDTA: Ethylene-diaminetetraacetic acid.

HP and IHC preparations were processed at the Research Center for Microscopic Morphology and Immunology of the UMPhCv. Microscopic images were studied on a Nikon Eclipse 55i microscope, equipped with a 5 Mp color CCD camera and the Image ProPlus 7 AMS software package (Media Cybernetics Inc., Buckinghamshire, United Kingdom). In our study, we used the following antibodies (Table 1).

Statistical study

In order to carry out the study, the sections were photographed in the regions of interest with a 40× objective, on a Nikon Eclipse E200 microscope, taking 10 images from each case. For the anti-cluster of differentiation (CD)34 antibody, we measured the diameters of the blood vessels of lung tumors. We did the same for the control cases and then compared the results and plotted them. For the quantification of the expression of CD68 and CD3, we calculated the integrated optical density (IOD) as the average intensity/density for each object (macrophage, T-lymphocyte) present on each image and averaged across cases. We compared cases of lung cancer with normal lung tissue and all values were graphically represented and interpreted using Microsoft Excel 10 and GraphPad Prism 9.2 (GraphPad Software LLC, Boston, MA, USA). Comparisons were then made for the two pathological

groups, namely the control, and the SCC of the lung casuistry utilizing a Student's *t*-test. Data were reported as mean ± standard deviation (SD). In all cases, $p<0.05$ was used to indicate statistical significance.

Results

Histopathological analysis

HP analysis showed that the tumors were formed of nests of polymorphic, atypical cells, predominantly polyhedral, with a tendency to keratinization, separated by more or less extensive stromal areas. The cells showed nuclear atypia, polymorphic, nucleolated nuclei, with abundant euchromatin and acidophilic cytoplasm (Figure 1). Areas of tumor necrosis were evident in the tumor structure.

By using the cytokeratin 7 (CK7) marker, we observed that tumor cells infiltrate alveolar spaces and terminal respiratory ducts with epithelium positive for CK7, while tumor cells are negative for CK7 (Figure 2). CK7 was positive in three out of 19 (15.7%) SCCs.

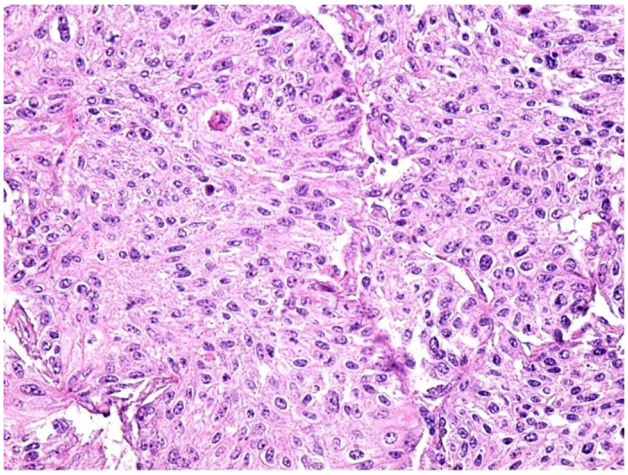

Figure 1 – ***Nests of squamous cells, with numerous nucleo-cytoplasmic atypia separated by fine areas of stroma. HE staining, ×200. HE: Hematoxylin–Eosin.***

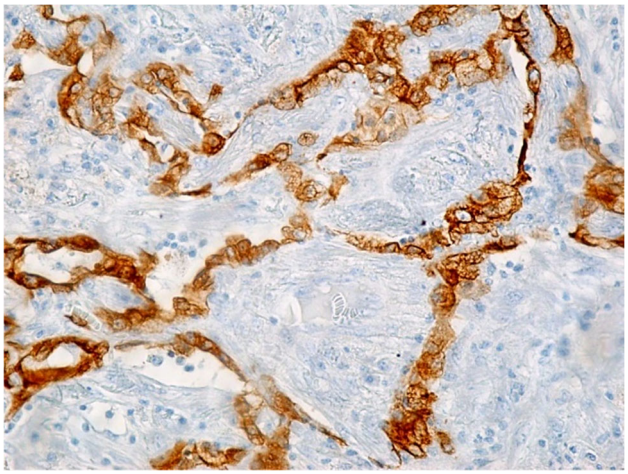

Figure 2 – ***Tumor cells infiltrating alveolar spaces and terminal respiratory ducts with epithelial positive for CK7, whilst the tumors cells are negative for CK7. Immuno-staining with anti-CK7 antibody, ×200. CK7: Cytokeratin 7.***

In some patients, the tumor cells were, almost entirely, positive for the anti-p53 antibody (Figure 3), which denotes an inactivation of the tumor protein p53 (*TP53*) suppressor gene. In the process of carcinogenesis, tumor cells manage to create a favorable environment, formed of fibroblasts, immune cells, blood vessels, and extracellular connective matrix, called the TME. The main cells of the connective stroma, including the tumor stroma, are fibroblasts. These cells are capable of synthesizing all the components of the tumor connective matrix. Most often they appear in repair processes and in carcinogenesis in the form of young cells, known as myofibroblasts. In our study, we identified myofibroblasts by using the mouse anti-human smooth muscle actin (anti-α-SMA) monoclonal antibody. Thus, we were able to highlight the presence of numerous myofibroblasts in the connective stroma (Figure 4) responsible for the synthesis of all biochemical components of the extracellular matrix (ECM).

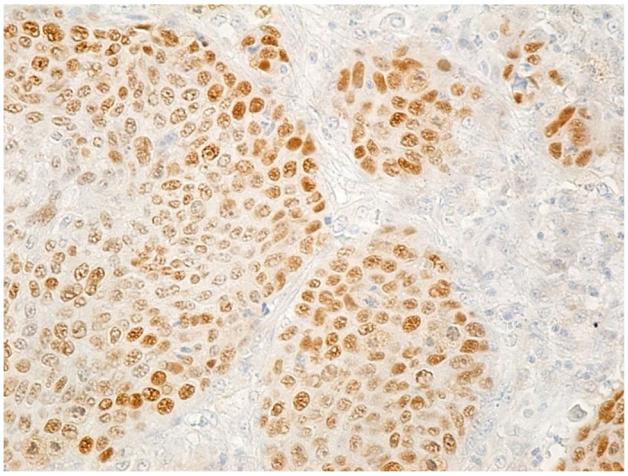

Figure 3 – ***Tumor cells with an intensely positive reaction to the anti-p53 antibody. Immunostaining with anti-p53 antibody, ×200.***

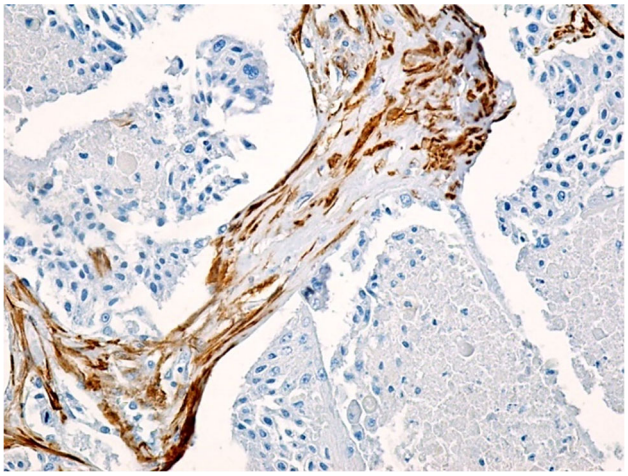

Figure 4 – ***Numerous myofibroblasts present in the tumor stroma. Immunostaining with anti-α-SMA antibody, ×200. α-SMA: Alpha-smooth muscle actin.***

The inflammatory infiltrate was of medium intensity, non-homogeneously distributed, formed of white blood cells (predominantly T-lymphocytes and macrophages). Most often, such cells were located perivascularly, showing their blood origin (Figure 5). A group of white blood cells involved in immune defense but also in carcinogenesis is

formed by the cells of the monocyte–macrophage system. Using the anti-CD68 antibody, we specifically marked macrophages. They were identified in large numbers in the peritumoral stroma, intratumoral stroma, but also among the carcinomatous cells (Figure 6). A large number of macrophages were identified in the areas of tumor necrosis (Figure 7). CD3-positive T-lymphocytes were identified in large numbers in the peritumoral stroma and in a smaller number in the intratumoral stroma and among the tumor cells (Figure 8). In contrast, B-lymphocytes were present in small numbers in the peritumoral stroma and almost absent in the intratumoral stroma (Figure 9).

Other cells with a major role in the genesis of the TME are endothelial cells; these are cells that line blood vessels, cells capable of dividing rapidly and forming new vessels that bring oxygen and plastic substances necessary for tumor development to the tumor cells. In our study, using the anti-CD34 antibody, we observed an increase in the number and caliber of intratumoral blood vessels (Figure 10). The arrangement of newly formed vessels was immediately below the basal membrane of the neoplastic cell islands.

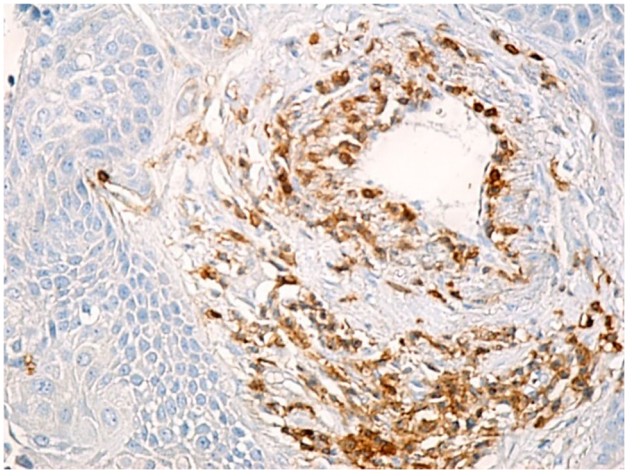

Figure 5 – ***Area of tumor stroma infiltrated with numerous white blood cells. Immunostaining with anti-CLA antibody, ×200. CLA: Cutaneous lymphocyte antigen.***

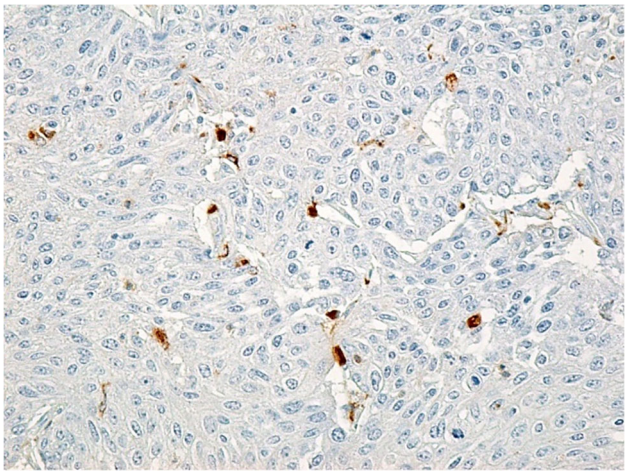

Figure 6 – ***Macrophages present in the tumor stroma, but also intratumorally. Immunostaining with anti-CD68 antibody, ×200. CD68: Cluster of differentiation 68.***

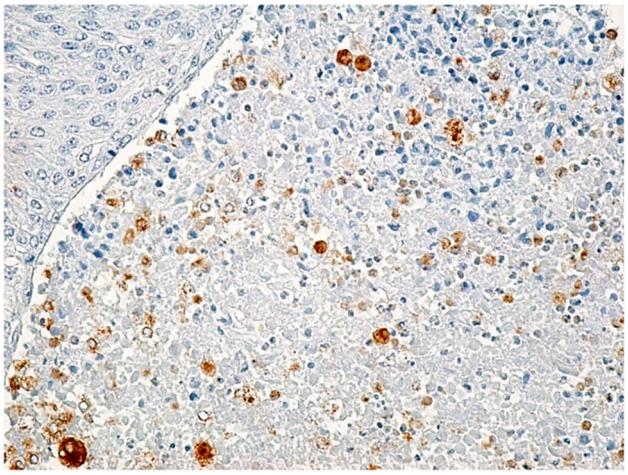

Figure 7 – ***Area of tumor necrosis, rich in macrophages. Immunostaining with anti-CD68 antibody, ×200.***

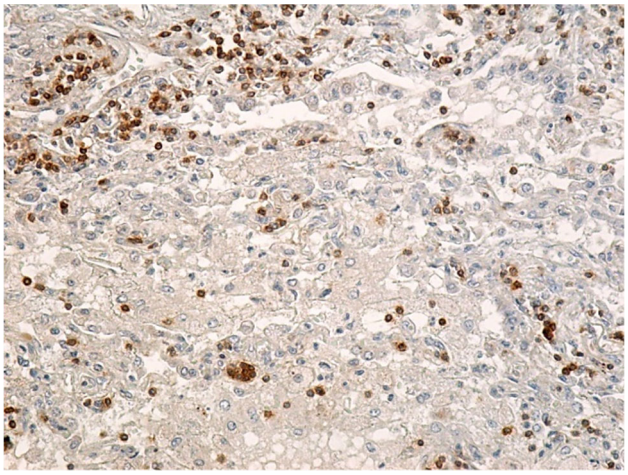

Figure 8 – ***CD3-positive T-lymphocytes present in the peritumoral and intratumoral stroma. Immunostaining with anti-CD3 antibody, ×200. CD3: Cluster of differentiation 3.***

Statistical study

In our study, we identified an increased number of macrophages in patients with SCC of the lung. For each case, we calculated IOD – the average intensity/density of the signal and compared the values from patients with lung carcinoma with the average IOD values obtained on control cases with normal lung tissue. In addition to the increased number of macrophages, there was an increase in their volume, which gave an intense reaction with the anti-CD68 antibody, which demonstrates the large increase in the number of lysosomes, the intensification of phagocytosis activity, the intensification of collagenase secretion that determines the remodeling of the extracellular microenvironment. Outlining phagolysosomes, with intracytoplasmic expression, has a clearly increased value compared to the control, but also a large variability (SD). It may suggest increased phagocytic activity associated with the tumor. Measurements were made intratumorally and juxtatumorally, areas of comedonecrosis were not included (Figure 11).

We did the same with anti-CD3 antibody (Figure 12), the results achieving a statistically significant difference (p=0.0263). Higher IOD values suggest a greater presence and expression of CD3-positive T-lymphocytes in cancer tissue.

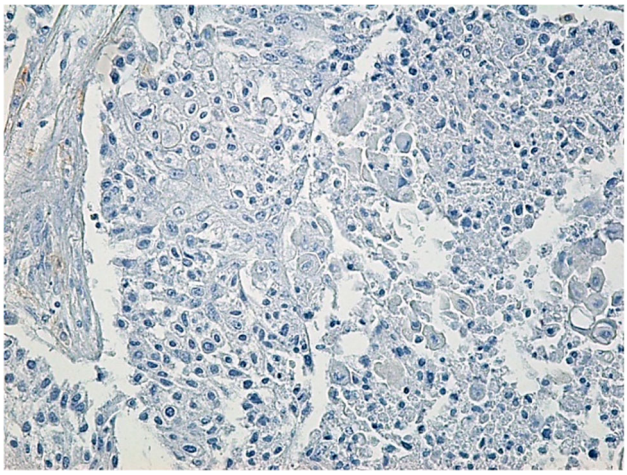

Figure 9 – ***B-lymphocytes absent in the tumor. Immunostaining with anti-CD20 antibody, ×100. CD20: Cluster of differentiation 20.***

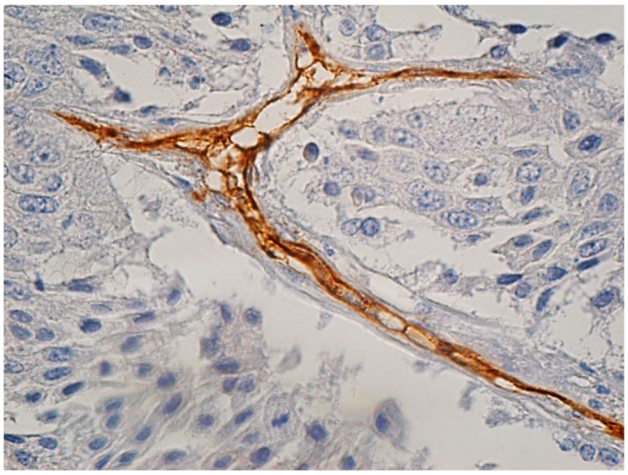

Figure 10 – ***Tortuous neoangiogenesis capillaries in the pulmonary stroma. Immunostaining with anti-CD34 antibody, ×400. CD34: Cluster of differentiation 34.***

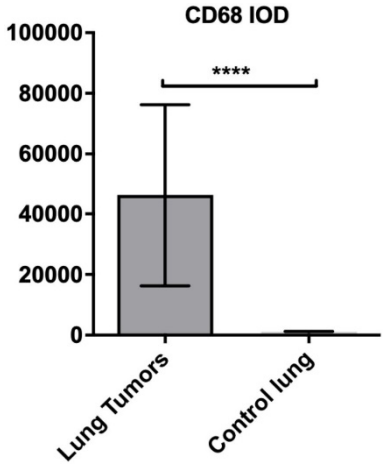

Figure 11 – ***IOD of CD68 in tumor and normal tissue. CD68: Cluster of differentiation 68; IOD: Integrated optical density.***

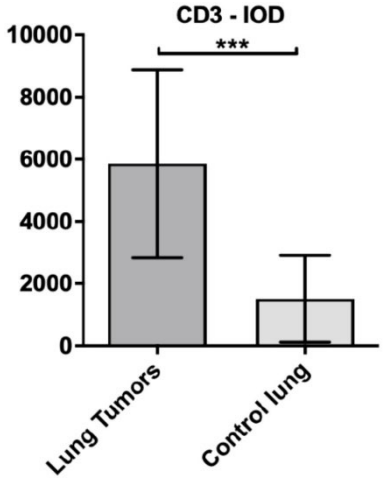

Figure 12 – ***IOD of CD3 in tumor and normal tissue. CD3: Cluster of differentiation 3; IOD: Integrated optical density.***

By supplying oxygen and nutrients required for tumor growth as well as by generating an acidic environment that may heighten tumor aggressiveness, tumor vascularization has a substantial impact on TME. CD34 is a marker for both vascular endothelium and hematopoietic progenitor cells, CD34 here shows both mature and newly formed vessels, having a granular-continuous appearance. We measured the diameters of blood vessels using the anti-CD34 antibody, which indicated that the mean blood vessel diameters in patients with lung cancer were much higher compared to the mean microvascular densities in control groups (Figure 13). The mean diameters of blood vessels in patients with lung cancer were approximately 10.5–13.25 μm, compared to the mean diameters of control lung tissues of 4.5–6.5 μm, substantiating a stronger vascularization in cancer tissue based on the increased tumor metabolic requirements.

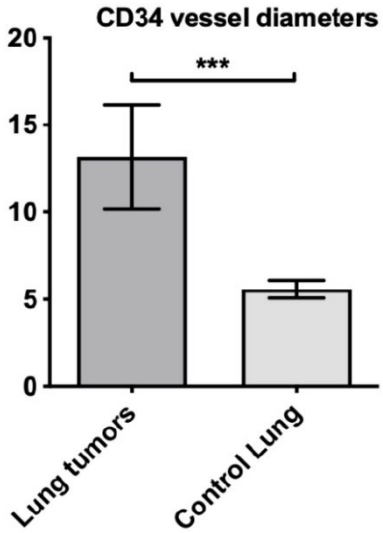

Figure 13 – ***Blood vessel diameter is significantly increased in lung cancer compared to normal lung tissue. Student's t-test: p<0.0001. CD34: Cluster of differentiation 34.***

Discussions

Lung cancer is a major health problem worldwide, as it is the leading cause of cancer death in both men and women [4]. SCC of the lung remains a leading cause of cancer deaths also due to its resistance to therapy. Pulmonary

carcinogenesis is a complex, multifactorial process that begins with pulmonary stem cells. The lung, like many other organs, contains several types of stem cells involved in the processes of repair and regeneration of lung tissue damaged by various etiopathogenic factors, for maintaining homeostasis of the respiratory system. These are: type II alveolar cells, bronchioalveolar stem cells, and basal cells of the respiratory epithelium. When these cells acquire genetic mutations, they can undergo malignant transformation, which helps them proliferate uncontrollably, acquire metabolic plasticity and resistance to the immune response, and generate various forms of tumors.

The process of pulmonary carcinogenesis is quite complicated and heterogeneous and includes genetic factors (gene insertions, deletions, and translocations), environmental factors, and epigenetic modifications [4, 14]. A key stage in the metastatic cascade is when cell adhesion is lost or malfunctions [15–19].

Stem cells are capable of differentiating into type 2 alveolar cells and Clara cells (non-ciliated secretory cells present in the bronchiolar epithelium) being essential for the normal functioning and regeneration of the alveolar and airway epithelium of the lungs. They play an essential role in the repair and regeneration of lung tissue exposed to inflammation and toxic agents, but also in the development of lung cancer [14, 20].

Epigenetic factors determine key mutations in some oncogenes such as: the *TP53*, Kirsten rat sarcoma virus (*KRAS*), phosphatase and tensin homolog (*PTEN*), *EGFR* genes, disrupting the normal signaling pathways that underlie abnormal cell proliferation. Mutation or deletion of p53 is a common genetic deficiency in lung cancer and other malignancies [21, 22]. P53 mutations are more common in bronchial carcinomas associated with smoking, particularly small-cell lung cancer (SCLC) and SCC. The prevalence is smaller in adenocarcinomas, where the link with smoking, while existent, is less robust. P53 anomalies are more common in people who have had more tobacco exposure in their lifetime. Tobacco-specific carcinogens produce a distinctive spectrum of p53 mutations that differ from those identified in non-smoking malignancies. This genetic "signature" may linger for decades after quitting smoking [23]. In our study, approximately 40% of the samples examined showed a positive reaction to the anti-p53 antibody, evidence of *TP53* gene alteration. According to some studies, mutations in *TP53* (a tumor suppressor gene, prevents basal cells from undergoing apoptosis or cell cycle arrest) in response to deoxyribonucleic acid (DNA) damage, allowing the accumulation of mutations that subsequently lead to the development of cancer [24–26]. The primary function of the EGFR is to promote cellular growth and development while simultaneously ensuring cellular survival [13, 27]. This factor has been studied in relation to its involvement in various pathologies, with lung cancer being one of the major ones [27]. Both the prevention and treatment of this type of cancer are directly influenced by the blockade of the EGFR signaling pathway [13].

Inflammation plays a pivotal role in the initiation, progression, and metastasis of tumors, with inflammatory cells significantly influencing tumor behavior [28, 29]. A key aspect of this process is the presence of inflammatory cells such as macrophages and tumor-infiltrating lymphocytes (TILs), which can either promote or suppress tumor growth depending on their state of activation and interaction with other cells.

Cancer evolution is closely associated with the surrounding TME [30]. This is a complex system formed of immune cells, inflammatory cells derived from bone marrow, blood vessels, fibroblasts, ECM, and signaling molecules [2, 31]. Immune cells that infiltrate tumors are key components of the TME and can have both anti-oncogenic and pro-oncogenic effects [32–34]. In the case of tumors, cancer cells develop several mechanisms to evade immune destruction, including the secretion of cytokines that directly suppress cytotoxic T-cells. Moreover, the accumulation of tumor-associated macrophages (TAMs) has been shown to be closely associated with a poor prognosis in some forms of cancer [35, 36].

Considering the fact that inflammatory cells are found in tumor biopsy samples and that tumors frequently originate in areas of persistent inflammation, it is clear that inflammatory cells are essential to the initiation and spread of tumors [37, 38]. Inflammatory cells are potent tumor promoters in the early stages of neoplastic development, fostering angiogenesis and genomic instability while establishing a favorable environment for tumor growth. Cancer is always accompanied by inflammatory processes, and chronic, ongoing inflammation in cancerous tissue leads to the suppression of anti-tumor immunity through a number of different pathways [39, 40]. Macrophages and TILs can either stimulate or inhibit the growth of tumors.

The most common non-cancerous cells linked to tumor progression are TAMs, which are crucial for carcinogenesis, neoangiogenesis, neurogenesis, immunosuppressive TME remodeling, recurrence, chemoresistance, and metastasis [41, 42]. Different microenvironments and stimuli can cause macrophages to polarize into different patterns. These cells are often classified into two groups: M1 macrophages, which are proinflammatory, and M2 macrophages, which are anti-inflammatory, based on their distinct activation states, activities, and cytokine production [43]. Cytokines such as interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), and IL-6 are released when the M1-like pathway is activated. This promotes proinflammatory response to protect against malignant tumor cells and pathogenic effects [44]. Conversely, IL-4 and IL-13 are activated by the M2 phenotype, and the elevated levels of CD206 in these cells increases their capacity for endocytosis [45]. M2-like macrophages secrete IL-10, transforming growth factor beta (TGFβ), and various other anti-inflammatory cytokines, which may be implicated in immunological control, angiogenesis, and the encouragement of T-helper 2 cell differentiation and progression of tumors [46]. Research revealed that TAMs could promote the development of the immunosuppressive environment and impair the activities of other types of immune cells, especially T-lymphocytes and natural killer cells [47]. In our study macrophages, identified by the anti-CD68 antibody, were found to be concentrated in areas of tumor necrosis and intratumoral stroma.

Macrophages, particularly M2 subtype, are crucial for promoting angiogenesis by secreting pro-angiogenic factors like vascular endothelial growth factor (VEGF), which stimulates endothelial cells to form new vessels. In our study, the use of the anti-CD34 antibody revealed an increase in the diameter of blood vessels in the tumor compared to normal tissue. This enhanced vascularization is crucial for providing nutrients and oxygen to the rapidly growing tumor cells, which helps sustain their aggressive proliferation. The increased vascular density also correlates with the elevated expression of endothelial markers, indicating that angiogenesis is actively contributing to the tumor's growth and metastasis.

Our findings also highlight the important role of T-lymphocytes (CD3-positive) in TME. The presence of T-lymphocytes in both peritumoral and intratumoral regions suggests an active immune response against the tumor. However, the interaction between immune cells and tumor cells is complex. The study of TILs has advanced in the last years, as it has been shown that immune cells have an impact on the clinical course of several solid tumors [48, 49]. There are studies that highlighted that the prognosis of patients with lung cancer was highly correlated with the percentage of peripheral lymphocytes. In clinical studies, the percentage of lymphocytes may serve as a stratification factor and an indicator for illness outcome [50]. TILs can be detected in around 25% of individuals with lung malignancies, particularly in weakly differentiated carcinomas and cancers with microscopic vascular invasion [51].

TME, therefore, is a dynamic and evolving environment, with macrophages contributing to both angiogenesis and immune regulation, and T-lymphocytes either combating or inadvertently supporting tumor growth, thus influencing the overall aggressiveness and metastatic potential of the tumor. To find new biomarkers and treatment targets, the role of TME in lung cancer should be investigated further, especially in relation to environmental exposures like tobacco smoke.

Conclusions

The findings from this study demonstrated that lung cancer tissues exhibited significantly higher vascular density and marked differences in inflammatory cell populations compared to normal lung tissues. Notably, the presence of CD34-positive blood vessels indicated a higher degree of angiogenesis within the tumor environment, which is often associated with tumor progression. Additionally, the increased density of CD68-positive macrophages and the distribution of CD3+ T-lymphocytes suggest an active immune response surrounding the tumor. As lung cancer continues to be a major cause of cancer-related death globally, further studies investigating the molecular and immune landscape of the TME are crucial for developing more effective and personalized treatment approaches.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

Microscopic images have been acquired in the Research Center for Microscopic Morphology and Immunology, University of Medicine and Pharmacy of Craiova, Romania (Manager: Laurenţiu Mogoantă, Professor, MD, PhD).

Authors' contribution

Ovidiu-Lucian Cîmpeanu and Andrei Osman equally contributed to the manuscript.

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Received: January 18, 2025

Accepted: April 12, 2025