Structural and functional characterization of vascular phenotype in lung cancer

Caratterizzazione strutturale e funzionale dei fenotipi vascolari nel tumore polmonare

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INTRODUCTION
1.1 LUNG CANCER

Cancer is the second cause of death worldwide and lung cancer is the most common cancer variety, indeed is documented 1.6 million new cases (12.7% of all new cancer cases) and 1.4 million deaths (18.2% of all cancer deaths) in 2008, with most patients dying with metastatic disease.

Lung cancer was estimated to cause 160,340 deaths in 2012 in the USA, accounting for 28 percent of all cancer deaths causing more deaths than the next three most common cancers combined (colon, breast and prostate).

Over 226,160 new cases are estimated to be diagnosed in 2012, accounting for almost 14 percent of all cancer diagnoses¹.

1.2 ETIOLOGY

Lung cancer is unique among all malignancies in having a single factor that accounts for a high percentage of the attributable risk. It has been estimated that active smoking is responsible for close to 90 percent of lung cancer cases. Other risk factors, both known and hypothesized, include exposure to certain industrial substances (9-15%), such as arsenic, some organic chemicals; radon and asbestos, particularly among smokers. Radiation exposure from occupational, medical, and environmental sources, pollutants in the air (1-2%), exposure to environmental tobacco smoke among nonsmokers and diet and nutrition have been also implicated.

In addition, accumulating evidence has demonstrated familial aggregation of lung cancer, with higher risks among relatives of probands after controlling for known environmental factors. Because of the interactions between exposures, the combined attributable risk for lung cancer can exceed 100 percent².

1.3 HISTOLOGY

At histologic analysis is possible to distinguish two major types of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Sometimes a lung cancer may have characteristics of both types, which is known as mixed small cell/large cell carcinoma.

Small cell lung cancer is less common, accounting for approximately 14 percent of all lung cancer cases. This type of lung cancer grows more quickly and is more likely to spread to other organs. Small cell lung cancer often starts in the bronchi and towards the center of the lungs.
Non-small cell lung cancer accounts for the remaining 85 percent of lung cancer cases. It generally grows and spreads more slowly than small cell lung cancer. The American Lung Association classified non small cell lung cancer as constituted by three main types of tumor, named for the type of cells in which the cancer develops: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma.

1.4 CLINICAL AND THERAPEUTIC ASPECTS

Patients with SCLC are generally treated with chemotherapy and concurrent radiation (if staged as limited disease) or chemotherapy alone (if staged as extensive disease). Instead, primary tumor with characteristic of NSCLCs are generally treated with surgery (or radiation if the patient is not a surgical candidate) with or without adjuvant chemotherapy. Patients with metastatic disease are generally treated with chemotherapy to improve quality of life, palliate symptoms, and improve survival.

In a minority of patients, a mutant epidermal growth factor receptor (EGFR) has become a validated therapeutic target and EGFR tyrosine kinase inhibitors (TKIs) are currently the first-line treatment options for these patients. However, ultimately these tumors develop resistance to these TKIs through various mechanisms. Another strategy that has been supported by preclinical studies has been dual inhibition of the EGFR pathway with both a TKI and Monoclonal Antibodies (MoAb). Combination therapy may be particularly advantageous in tumors with wild-type EGFR, where EGFR activation is dependent on ligand binding. Mutant EGFR, however, can lead to constitutive activation of the EGFR, not requiring the presence of the ligand.

These considerations associated to the awareness that the formation of new blood vessels, as source of oxygen and nutrients for proliferation, is essential for tumor growth and is a limited process in healthy adults, has yielded angiogenesis a rational target for cancer therapy. The development of new therapeutic strategies designed to inhibit tumor-induced angiogenesis should prevent the growth of solid tumors and metastasis holding great promise for the advancement of lung cancer management.

2 TUMOR ANGIOGENESIS

The term “angiogenesis” is generally applied to the sprout of microvessels having the size of capillaries, a process that is orchestrated by a very sensitive interplay of growth factors and inhibitors.
The process of growing new blood vessels is a fundamental biological mechanism essential for development, reproduction, organ growth and repair. Outside of female reproductive cycles, angiogenesis in the adult is largely controlled by pathological situations, such as wound healing and tumor growth. Indeed, angiogenesis appears among the essential “hallmarks” or processes required for the transformation of a normal cell to a cancer cell.

The growth and development of solid tumors is critically dependent on a functional vascular supply and also their size and aggressiveness depend on their vascularization, in the absence of which tumors remain dormant and unable to metastasize.

In 1971, Dr. Judah Folkman published a landmark article in the *New England Journal of Medicine*, with the hypothesis that solid tumors caused new blood vessel growth in the tumor microenvironment by secreting proangiogenic factors. This publication heralded the beginning of research on angiogenesis and hypoxia and their role in cancer. Over the last 4 decades, the discovery of a plethora of genes, signaling cascades, and transcription factors has revealed the complexity of the angiogenic process and furthered our understanding of this hypothesis.

It is assumed that, initially, the growth of a tumor is fed by nearby blood vessels. Once a certain tumor size is reached, these blood vessels are no longer sufficient and new blood vessels are required to continue growth.

Initiation of angiogenesis can occur at different stages of the tumor progression pathway depending on the type of tumor and the environment and is believed to be reliant on an angiogenic “switch,” which leads to a complex series of events, starting with the release of tumor-related proangiogenic factors, endothelial cell activation, and the release of proteolytic enzymes, followed by endothelial cell migration, proliferation, and capillary tube formation.

Tumor angiogenesis differs from physiological in the imbalance between positive and negative controls. One characteristic feature of tumor blood vessels is that they fail to become quiescent, enabling the constant growth of new tumor blood vessels. Consequently, the tumor vasculature develops unique characteristics and becomes quite distinct from the normal blood supply system.

The tumor vasculature usually consists of disorganized, leaky, premature, torturous, and hemorrhagic blood vessels that provide a structural basis for cancer cell invasion and spread. So, the structural and functional abnormalities in tumor vasculature reflect the pathological nature of their induction.
2.1 Angiogenesis Regulation

Control of the angiogenic switch is reliant on how heavily the balance of pro- and antiangiogenic factors tips in favor of pro-angiogenesis. Acquisition of the angiogenic phenotype can result from genetic or local environmental changes that lead to the activation of ECs. One way for a tumor to activate ECs is through the secretion of proangiogenic growth factors, which then bind to receptors on nearby dormant ECs that line the interior of vessels. At the time of EC stimulation, vasodilation and permeability of the vessels increase, and the ECs detach from the extracellular matrix and basement membrane by secreting proteases known as matrix metalloproteinases. The ECs then migrate and proliferate to sprout and form new branches from the pre-existing vasculature. The growth factors can also act on more distant cells recruiting bone marrow-derived precursor ECs and circulating ECs to migrate to the tumor vasculature. The proangiogenic growth factors may be overexpressed because of genetic alterations of oncogenes and tumor suppressors, or in response to the reduced availability of oxygen. Vascular endothelial growth factor (VEGF) and its Tyrosine Kinase Receptor (VEGFR) play key roles in angiogenesis.

2.1.1 Role of VEGF in angiogenesis regulation

Although VEGF is actually a family of at least 7 members, the term VEGF typically refers to the VEGF-A isoform. VEGF has been identified as the most potent and specific mitogen for endothelial cells, with a key role in activating the angiogenic switch. VEGF is also responsible for endothelial cell survival signaling in newly formed vessels and induces significant vascular permeability, resulting in the highly permeable vessels that are characteristic of tumor vasculature. As with many tumor types, high VEGF levels have been correlated with poor prognosis in patients with lung cancer and high VEGF levels have been identified as an independent prognostic factor. Many of the most successful antiangiogenic agents are therefore those aimed at inhibiting the proangiogenic effects of VEGF.

2.1.2 Role of PDGF in angiogenesis regulation

The family of PDGF ligands is comprised of 4 structurally related, soluble polypeptides (PDGF-A, PDGF-B, PDGF-C and PDGF-D) that exist as 5 different homodimers and heterodimers.
There are 3 forms of the PDGF tyrosine kinase receptors: PDGFR-α and PDGFR-β and PDGFR-αβ. PDGF signaling indirectly regulates angiogenesis by inducing VEGF transcription and secretion. The family of platelet-derived growth factors (PDGFs) and receptors (PDGFRs) are involved in vessel maturation and recruitment of vascular mural cells: pericytes and vascular smooth muscle cells (VSMCs). PDGF is expressed by endothelial cells and generally acts in a paracrine manner, recruiting PDGFR-expressing cells, particularly pericytes and smooth muscle cells, to the developing vessels. Deletion of PDGF and PDGFR in mice results in hemorrhage and tissue edema in early embryos as a consequence of defective vascular development. Mutations involving upregulation of PDGF and/or PDGFR have been described in human cancers, indicating a likely role for the PDGF pathway in carcinogenesis.

### 2.2 Role of Lymphatic Vessels

In the last two decades a large number of clinical studies have indicated that the angiogenic pattern may be a useful indicator of clinical outcome of tumors. As with all solid tumors, the role of angiogenesis is well established in the progression of lung cancers, and much data has shown that high microvessel density, as an index of neo-angiogenesis, has been identified as a prognostic factor predictive of metastasis and poor survival. In particular, high vascularity at the tumor periphery has been correlated with tumor progression.

A considerable body of research spanning almost three decades has documented that angiogenesis plays an important role not only in the tumor growth and its blood supply, but also in the metastatic process. Although angiogenesis alone is not sufficient for developing metastases, new blood vessel formation increases the opportunity for malignant cells to enter the blood stream and thus metastatize. Newly formed capillaries are permeable because of fragmented basement membranes, making them more accessible to errant tumor cells. The cancer cell migration to distant tissue occurs through blood and lymphatic vessels. However, preclinical experimental systems supported by clinical evidence suggest the most common pathway of initial metastasis is through the lymphatic system.

Historically, lymphatic vessels were considered passive participants in tumor metastasis by simply providing channels for tumor cells to transit through. However the discovery of several key lymphatic- specific molecular markers and an increased availability of *in vitro* and *in vivo*
Experimental systems to study lymphatic biology have highlighted a much more complex, active role for the lymphatic vasculature in metastatic tumor spread. A great number of studies have investigated the role of lymphangiogenic factors in human cancer metastasis primarily via immunohistochemical analysis of tumor tissue samples and retrospective comparison to patient outcomes. A number of studies have indicated that increased lymphatic vessel invasion significantly increases the risk of lymph node metastasis, distant metastasis, and death.

### 3. CANCER INITIATING CELLS (CICs) AND VASCULAR NICHE

Recently it was introduced the concept of cancer initiating cells (CICs). Indeed, increasing evidence indicates the existence of small subpopulations of cells endowed with unique self-renewal capacity, tumorigenesis, and radio- and chemotherapy resistance, defined as cancer stem cells (CSCs). These cells are predicted as a critical population of tumor cells which might mainly drive each step of tumor programming, including neoplasm initiation, tumor growth and metastasis. Initially, cancer stem cells were identified in hematological malignancies, then they were isolated subsequently from many organs including breast, prostate, brain, gastrointestinal, liver, lung, skin and ovarian cancer. Recent discoveries revealed the interplay among CSCs, angiogenesis and tumor vasculature.

Cancer stem cells show greater potential for tumor initiation and formation than non-stem cell like tumor cells. Although VEGFs are expressed at elevated levels by endothelial cells engaged in angiogenesis and circulating bone marrow derived endothelial progenitor cells, many types of human cancer cells can also secrete VEGFs. In these regards, it is logical to suggest that cancer stem cells can play a pivotal role in angiogenesis. Recently, an emerging area of research supports that CSCs, besides their self-renewal and proliferative capabilities, may promote tumor angiogenesis expressing angiogenic factors to the cancer microenvironment.

Both stemness and angiogenesis associated factors might be potential biomarkers for clinical prediction.

The first notion of tumor microvascular-environment goes back to 1940s, when Scherer suggested that glioblastoma cells grow into the site which is surrounded by blood vessels. From then on, abundant reports support the theory that the neo-vascularity formed around tumors play a special role in cancer development and metastasis. Recently it was called the ‘cancer vascular niche’.
Current CSC research generates a large amount of data that show the relationship between cancer stem cells and the cancer vascular niche.

As mentioned above, cancer stem cells express vascular-related agents in order to induce neovascular growth and establish an aberrant vascular niche. On the other hand, tumor angiogenesis and formation of a cancer vascular niche contribute to maintenance and further proliferation of cancer stem cells.

Further research on the interaction of cancer stem cells and angiogenesis will help to develop some novel therapeutic strategies against cancer as a bilateral benefit. Regarding the close interaction between CSCs and tumor angiogenesis it seems so far reasonable to combine traditional radio- and chemotherapy with anti-angiogenic therapy\textsuperscript{36}.

Since CSCs are associated to therapy resistance and disease recurrence, the development of clinically useful inhibitors to target the individual CSC niche should be prioritized\textsuperscript{39,40}. 

\textit{Introduction}
4. AIM OF THE STUDY

Despite advances in treatment, the prognosis for lung cancer patients remains poor. Angiogenesis control appears to be a promising target for lung cancer therapy since it represents a crucial step in cancer progression playing an important role in tumor growth and metastasis. Moreover blood and lymphatic vessels closely interact with Cancer Initiating Cells (CICs) as they constitute the vascular niche.
Thus, the aim of the present study was to characterize at tissue level the structural rearrangements of blood and lymphatic vessels in human lung cancer. The incidence of vascular progenitor cells through the evaluation of PDGF- receptor expression was also investigated.
In addition, the in vitro angiogenic and cell migration ability was tested on vascular phenotypes isolated and expanded from normal and neoplastic human lungs.
MATERIAL AND METHODS
1. Case Study And Tissue Sampling

The study was performed on 13 patients affected by lung cancer, admitted to the Unit of Thoracic Surgery, University of Parma, undergoing lung resections, enrolled after informed consent to the employment of biologic samples for research purpose.

Lung tissue was collected and transported under sterile condition to the Department of Pathologic Anatomy, where it was sampled under hood at laminar flow.

Portions of neoplastic or healthy lung tissue were sampled by the medical staff ensuring the priority of their use for diagnostic purposes. The correct sampling procedure was confirmed by the subsequent histologic analysis.

Lung tissue obtained from neoplastic and healthy portions was put in sterile PBS in order to perform cell isolation or fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemical analysis.

In order to evaluate tissue feature and to identify cellular population of interest, these samples were compared to a control group (CTRL) represented by 12 lungs collected from patients lacking pathologic states.

2. In Vivo Study

2.1 MORPHOMETRIC ANALYSIS

After paraffin inclusion, from each sample serial sections of 5 µm thickness were stained with Haematoxylin and Eosin (H&E), Masson’s Trichrome or used for immunohistochemistry.

On lung tissue, obtained from patients, utilized both for cell isolation and histologic assessment, was performed morphometric and immunohistochemical analysis, sharing samples in tumoral portion (T) and distal area (Dist), represented by parenchima at least 5 cm far from neoplasia.

In particular, morphometric analysis was performed by an optical microscope in order to evaluate the volume fraction of alveoli and the volume fraction occupied by cancer cells.

In detail for each section stained with H&E a quantitative evaluation of tissue composition was performed measuring area occupied by alveoli and pneumocytes or by cancer cells on macroscopic images captured by Fotovix connected to Image Pro-Plus 4.0 software (Media Cybernetics, Bethesda, MD, USA). To define the volume fraction of alveoli and the volume fraction of tumor, the area represented by this tissue components was expressed as percentage of the total area explored.
**Material and Methods**

The volume fraction of fibrosis was calculated on Masson’s Trichrome stained sections at 250X magnification with the aid of a grid defining a tissue area of 0.23 mm² and containing 42 sampling points each covering an area of 0.0052 mm² on a large number of adjacent fields (>100 for each section). To define the volume fraction of fibrosis, the number of points overlying replacement and perivascular pulmonary fibrosis was counted and expressed as percentage of the total number of points explored.

### 2.2 IMMUNOHISTOCHEMICAL ANALYSIS

The immunohistochemical analysis was conducted on fluorescence and optical preparations. A first phase of preparation is common to the two technique: deparaffination in Xylene and in a decreasing concentration of ethanol, rehydration and antigenic unmasking. The antigen retrieval consists on pre-treatment in citrate buffer (pH=6) by microwave (900Watt, 3 cycle 5’ each) or with water bath (pH=9, 70°C, 40’).

#### 2.2.1 Immunofluorescence Technique

This method is based on the specific recognition of an antigen-antibody reaction. Once incubated with the specific primary antibody, the reaction is revealed by incubation with the secondary antibody conjugated to a fluorochrome (Fluoresceine Isothiocyanate- FITC, Tetramethyl Rhodamine Isothiocyanate -TRITC or Cyanide 5 -CY5). The nuclear counterstaining is performed with DAPI (4’,6-diamidine-2-phenyndole, Sigma St. Louis, MO, 5mM, 18’ RT). Slides were mounted with fluorescence mounting medium.

#### 2.2.2 Immunoperoxidase Technique

This method uses peroxidase as reaction catalytic enzyme that requires the previously inhibition of endogen peroxidases made by dipping the sections into a 3% H₂O₂ solution for 10 minutes. Once incubated with the specific primary antibody, the sections were incubated with the secondary antibody able to recognize the Fc fraction of the primary antibody in a species-specific manner. The reaction was revealed using peroxidase-conjugated streptavidin that catalyze the oxido-reduction reaction of the chromogenic substrate (DAB, 3-3’ diaminobenzidin for 5 minutes). All sections were counterstained with Haematoxylin and Eosin.
2.2.3 Blood and lymphatic vessels distribution

Five-micrometer-thick sections obtained from each group of patients were analyzed to determine blood and lymphatic vessels distribution in order to evaluate the circulatory system rearrangement in presence of this pathology.

To this purpose was detected the positivity for CD34, an antigen that highlights a transmembrane glycoprotein expressed on the surface of progenitor cells of lymphoid and hematopoietic human lineage, as well as endothelial cells and the α-SMA antigen, a fetal isoform expressed by arteriolar vessels. Histological samples were incubated with primary antibody anti CD34 (1:500, 60’, 37°C, clone QBEND/10, Neomarkers, Freemont, CA, USA). The reactions was revealed by diaminobenzidin (DAB) following the advance HRP method (DAKO) for the visualization in optic microscopy.

Capillaries were identified also by expression of von Willebrand factor antigen. In detail, samples were incubated with primary antibody (vWF, 1:30, 90’, 37°C, Sigma Aldrich, St. Louis, MO, USA) and subsequently with anti-rabbit TRITC conjugated secondary antibodies (1:20, 60’, 37°C, Jackson Laboratory, Baltimore, PA, USA). Nuclei were counterstained by DAPI (4’,6-diamidine-2-phenyndole, Sigma-Aldrich) and cover slips mounted with Vectashield (Vector, USA) and sections analyzed using a fluorescence microscope (DMI6000B Leica, 630X final magnification).

To detect the presence of α-SMA antigen histological sections were incubated with primary antibody anti α-SMA (Mouse monoclonal, 1:20, 90’, 37°C, Sigma Aldrich, St. Louis, MO, USA) and revealed by TRITC-conjugated anti-mouse secondary antibody (1:20, 60’, 37°C, Jackson Laboratories, Baltimore, PA, USA). Nuclei were counterstained by DAPI.

The quantification of capillaries, venules and arterioles vessels was performed both on T and Dist portion, using an optic (Zeiss 433048) or a fluorescence microscope (Olympus BX60) at the final magnification of 400X to calculate percentage and density of cells and structures expressing different antigens. In details the immunoreactions for CD34 allowed to evaluate and quantify the distribution of capillaries (diameter <8µm) and high diameter vessels (venules= 8-40µm).

One section for each sample was incubated with monoclonal mouse D2-40 epitope of podoplanin (Pdpn) antibody (1:50, o.n., 4°C, Biocare, Concord, CA, USA). Sections were then incubated with anti-mouse FITC conjugated secondary antibodies (1:20, 60’, 37°C, Jackson Laboratory, Baltimore, PA, USA) and analyzed using a fluorescence microscope (DMI6000B Leica, 630X final magnification) to calculate percentage and density of specific structures expressing the antigens. Pdpn is exclusively expressed by lymphatic vessels, allowing the possibility to distinguish lymphatic and blood vessels.
2.2.4 Vascular progenitors

Abundant evidence indicates that Plateled Derived Growth Factor (PDGF) and its receptors (PDGFR) are implicated in angiogenesis\textsuperscript{41,42,43}. In order to evaluate the incidence of PDGFR positive cells, immunohistochemistry has been performed incubating sections with anti PDGFR-β (monoclonal rabbit antibody, 1:100, o.n. 4°C, Abcam, Cambridge, UK) primary antibody, revealed by FITC-conjugated anti-rabbit secondary antibody (1:20, 60’, 37°C, Jackson Laboratories).

Samples were also incubated with primary antibody anti α-SMA (Mouse monoclonal, 1:20, 90’, 37°C, Sigma Aldrich, St. Louis, MO, USA) or anti Pan-Citokeratin (Mouse monoclonal, 1:50, 90’, 37°C, DAKO, Glostrup, Denmark) revealed by TRITC-conjugated anti-mouse secondary antibody (1:20, 60’, 37°C, Jackson Laboratories, Baltimore, PA, USA) in order to document the perivascular or peritumoral localization of PDGFR-β\textsuperscript{pos} cells.

PDGFR-stained sections were analyzed with fluorescence microscopy (Olympus, 400X Final Magnification) in order to determine PDGFR\textsuperscript{pos} cells density (n/mm\textsuperscript{2}).

In addition, since it is known that CD34 is expressed by vascular progenitor cells, individual CD34 positive cells were also computed.

2.3 STATISTICAL ANALYSIS

The SPSS statistical package was used (SPSS, Chicago, IL, USA). Normal distribution of variables was checked by means of the Kolmogorov-Smirnov test. Statistics of variables included mean ± standard error (S.E.M.), paired Student t-test, one-way analysis of variance (post-hoc analyses: Bonferroni test or Games-Howell test, when appropriate). Statistical significance was set at p<0.05.
3. In Vitro Study

3.1 ENDOTHELIAL CELL ISOLATION

Lung tissue fragments of about 1-2 gr., obtained from lung cancer or at sites distal from the tumors, were separately processed following the same protocol\textsuperscript{44,45,46}. The biologic material was minced using surgical scissors and the micro-fragments were put in a solution of collagenase/dispase (1mg/ml) for 75 minutes in a shaking bath at 37°C. The digest was then purified from debris using a nylon filter with pores of 100µm. The cell suspension was centrifuged at 240g for 5 minutes. The pellet was then resuspended in culture medium EGM-MV (Lonza, Basel, Switzerland) added with 10% FBS and VEGF-C (10ng/ml, Reliatech, Wolfenbuttel, Germany) and seeded in collagen-coated 6 well plates. Twenty-four hours after plating the debris and the non-adherent cells (erythrocytes, leucocytes, death cells) were removed by washing twice with PBS; fresh culture medium was then added to cell culture. Cell monolayer was daily observed using an inverted microscope (Olympus CK40, Japan) and fresh culture medium was changed twice a week. Within 5 days was observed an heterogeneous cell population mainly constituted of fibroblasts, stromal, epithelial and endothelial cells.

3.1.1 Hematic and Lymphatic Endothelial Cell Enrichment

In order to obtain a cell population mainly constituted of endothelial cells isolated from healthy lung tissue or from lung cancer, a sorting, using immunomagnetic beads, was performed. Briefly, the heterogeneous cell population was detached by Trypsin/EDTA (Sigma Aldrich) and resuspended in 60µl of EGM-MV, added with 20µl of FcR Blocking Reagent and 20µl of paramagnetic microbeads covered with antibodies against the CD31 antigen (Myltenyi Biotec.). After 20 minutes of incubation at 4°C, the cell suspension was transferred in a MS Column exposed to a magnetic field produced by OctoMACS SeparatorTM and then eluited with 2ml of EGM-MV. The cell population thus sorted consists of all the cells that don’t express the endothelial marker CD31. Afterwards, the MS Column was removed from the OctoMACS SeparatorTM and eluited with 2ml of EGM-MV in order to recover the CD31\textsuperscript{pos} cell fraction.
Material and Methods

The CD31^pos^ cell population was seeded in a collagen-coated 25cm^2^ flask with culture medium EGM-MV added with 10% FBS and 10ng/ml of VEGF-C. After 5-7 days an homogeneous cobblestone-like cell monolayer was observed. Lung tissue is perfused by both hematic or lymphatic vascular system, so the CD31^pos^ endothelial cell population was further sorted for Podoplanin, a specific marker of Lymphatic endothelial cells. The CD31^pos^ cell cultures, isolated from human lung tissue and from lung cancer, once reached 80% confluence, were detached by Trypsin EDTA (Sigma Aldrich) and centrifuged at 240g for 5 minutes. Cells were then resuspended in 100µl of PBS added with FBS (0,5%) and incubated for 25 minutes at 4°C with mouse-anti Human Podoplanin antibody (Reliatech). Cells were thus washed in PBS, centrifuged at 240g for 5 minutes, resuspended in 30µl of PBS added with FBS (0,5%) and incubated for 15 minutes at 4°C with anti-mouse IgG MicroBeads (Myltenyi Biotec). The cell suspension was then transferred in a MS Column exposed to a magnetic field produced by OctoMACS SeparatorTM and eluited with 2ml of PBS + 0,5% FBS. The cell suspension thus obtained was CD31^pos^/Podoplanin^neg^ and represented the Hematic Endothelial Cell population isolated from distal lung (BEC) and from lung cancer (T-BEC). Afterwards, the MS Column was removed from the OctoMACS SeparatorTM and eluited with 2ml of PBS + 0,5% FBS in order to recover the Podoplanin^pos^ cell fraction. Lymphatic endothelial cells (CD31^pos^/Podoplanin^pos^) isolated from human lung (LEC) and from lung cancer (T-LEC) were seeded in a collagen-coated 75cm^2^ flask with culture medium EGM-MV added with 10% FBS and 10ng/ml of VEGF-C and placed at 37°C-5% CO_2^ atmosphere.

3.2 IMMUNOCYTOCHEMICAL ANALYSIS

In order to evaluate the pureness of the CD31^pos^/Podoplanin^neg^ and CD31^pos^/Podoplanin^pos^ cell population an immunocytochemical analysis was performed. The cells cultured on suitable chamber slides were fixed with 4% paraformaldehyde, blocked, and permeabilized. The samples were then stained with primary antibodies anti-CD31 (mouse monoclonal, prediluited 4°C o.n., DAKO), anti-Podoplanin (rabbit, 1:100 4°C o.n., Reliatech)^47^ and anti-Lymphatic Vessel Endothelial Hyaluronan receptor-1 (LYVE-1, rabbit polyclonal, 1:20 4°C o.n., Abcam)^48^.

The expression of the nuclear transcription factor Prospero homebox protein-1(Prox-1, rabbit polyclonal,1:20 4°C o.n., Acris Antibodies, Herford, Germany)^49^ was also detected.
Material and Methods

The presence of the surface antigens was revealed through the conjugation with secondary antibodies anti-mouse and anti-rabbit respectively (FITC, 1:70 60’ 37°C, Sigma-Aldrich). TRITC-conjugated secondary antibody (anti-rabbit, 1:70 60’ 37°C, Sigma-Aldrich) was employed for the evaluation of Prox-1 expression. On the same cell lines the expression of tyrosine kinase receptors VEGFR3 (rabbit polyclonal, 1:20 4 °C o.n., Abcam) and PDGFRβ (Plateled Derived Growth Factor Receptor β, rabbit monoclonal, 1:20 4 °C o.n., Abcam)\textsuperscript{50,51,52} was investigated. The reactions with primary antibodies were visualized by incubation with fluoresceinated secondary antibody (goat anti-rabbit FITC, 1:70 60’37 °C, Sigma-Aldrich).

The presence of ki67 antigen was detected to quantify the incidence of cells in active cell-cycle. Briefly, lung sections were incubated with anti-ki67 primary antibody (mouse monoclonal, 1:50 4°C o.n., Novocastra, Newcastle Upon Tyne, UK) and then conjugated with anti-mouse secondary antibody (FITC, 1:70 60’ 37°C, Sigma-Aldrich). Nuclei were counterstained by DAPI and cover slips mounted with Vectashield (Vector, USA). The quantitative assessment of these markers was expressed as a percentage of positive cells.

3.3 MATRIGEL ASSAY

In order to evaluate endothelial cell tube formation, 100 µl of the Matrigel® matrix solution was transferred into each well of a 96-well plate. The matrix was allowed to solidify at 37°C and 5% CO\textsubscript{2} for 45 minutes. The endothelial cells were then trypsinized and seeded (2 × 10\textsuperscript{4} cells/well) in 100 µl fresh medium.

After 2, 5, and 24h of incubation, the tubular structures were visualized and photographed under an inverted light microscope at 4x magnification (Olympus, Japan). Quantitative assessment of the tube formation was carried out by using the Image Pro-Plus 4.0 software (Media Cybernetics, Bethesda, MD, USA) by measuring the area of the tubular structures. This area was divided by the total frame area to obtain the quantitative measurement of tube forming ability.
3.4 CELL MIGRATION ASSAY

Endothelial cells isolated from healthy human lung and from lung cancer were seeded (20000 cell/cm²) in 6-well plates. After confluence, cells were starved by adding Endothelial Basal Medium (EBM, Lonza, Basel, Switzerland) added with 0.5% FBS. After 12 hours of starvation a wound was made with a 1000µl pipette tip and usual culture medium with or without VEGF-C was added.

The closure of the wound area by migrating cells, if any, was observed at 0, 6 and 24 hours using an inverted-phase contrast microscope (Olympus CK40, Japan) coupled with a camera and images were recorded at each time point. The images were analyzed by Image Pro-Plus 4.0 software (Media Cybernetics, Bethesda, MD, USA). The percentage of open wound area was measured and compared with the value obtained at 0 hours. A decrease of the percentage of open wound area indicated migration of cells to cover the open wound area.
RESULTS and DISCUSSION
1. In Vivo Study

1.1 CASE STUDY

The patient population consisted of 13 subjects, 7 males and 6 females, aged between 54 and 72 years undergoing lobectomy for lung cancer. All patients had pulmonary malignancy, 4 of them had already been subjected to biopsy and the cryostat extemporaneous examination had confirmed the malignant lesion. The smoking status, type of intervention, diagnosis and histopathology of the disease, are shown in Table 1. The diagnosis performed by pathologists documented 7 Adenocarcinoma, 3 Squamous cell carcinoma and 3 Neuroendocrine tumors samples, as reported in Table 1.

Twelve lungs from patients lacking pathologic conditions were used as control (CTRL).

Table 1: Patients Population

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Sex</th>
<th>Age</th>
<th>Smoker</th>
<th>Type of intervention</th>
<th>Histological Diagnosis</th>
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1.2 MORPHOMETRIC EVALUATION OF FIBROSIS

In order to evaluate tissue composition of pathologic and control lungs, a morphometric evaluation was performed on histological section stained with Masson’s Trichrome. This analysis documented an increase in collagen accumulation in distal (18.73± 1.40%) and neoplastic (19.64± 1.67%) lung area when compared to control samples (7.14± 0.97%), as reported in Figure 1. Since
no difference was detected between the three different cancer histotypes, data were pooled and reported as neoplastic or distal portion of the lung.

The main form of collagen accumulation was replacement fibrosis since perivascular fibrosis was very limited.

1.3 IMMUNOHISTOCHEMICAL ANALYSIS

1.3.1 Vascular Structures

To evaluate the possible rearrangements of vascular structures in Lung Cancer, the morphometric measurement of capillaries, venules and arterioles was assessed. To this purpose, endothelial and smooth muscle cells were detected by immunohistochemistry with specific antibodies directed to CD34, von Willebrand Factor and α-SMA, respectively (Figure 2A-B and 3A-B).

In Distal (Dist) and Neoplastic (T) portions of the lung, the quantitative estimation indicated, respectively, a 77% and 74% increase in capillary density compared to CTRL lung. Conversely, the numerical incidence of CD34$^{\text{pos}}$ venules did not show a significant variation compared to CTRL (Figure 2C-D). The capillary and venule density was equally represented in the three different tumor histotypes, so that data were grouped.

A significant increase in the density of arterioles was observed in Dist and T areas of the lung in all tumor types compared to CTRL. In Adenocarcinoma and Squamous Cell Carcinoma samples, arteriolar density within the tumor tended to increase compared to the distal portion, however, only in Neuroendocrine carcinoma a significant 3-fold higher arteriolar density was measured in neoplastic tissue compared to the distal portion of the lung (Figure 3C).

Since the lymphatic system is an important determinant of tumor progression, podoplanin labeled vessels were analysed. The trend of lymphatic vessels density was similar in all types of cancer, so cumulative data are reported. It is important to point out that lymphatic vessels density was more abundant in distal lung parenchyma than within the neoplastic tissue. Quantitatively, a 42% and 94% decrease was observed, respectively, in distal and neoplastic lung compared to CTRL (Figure 4).

1.3.2 Vascular Progenitors

The role of PDGFs and its receptors in vessel maturation and recruitment of vascular mural cells prompted us to investigate the incidence of PDGFR-β$^{\text{pos}}$ cells to assess the distribution of
vascular progenitors in lung cancer (Figure 5A-B). To this purpose, we also detected individual cells expressing CD34, an antigen associated to hematopoietic and endothelial progenitors phenotype.

Pericytes, pseudovascular profiles and individual cells labeled by antibodies against PDGFR were detected by immunofluorescence (Figure 5A-B). Compared to CTRL lung, the numerical incidence of individual PDGFR$^\text{pos}$ cells was reduced in area of the lung distal (Dist) from tumor whereas increased within the neoplastic tissue. Quantitatively, in Dist from Adenocarcinoma and Neuroendocrine carcinoma, PDGFR$^\text{pos}$ cell density decreased by 64% and 61%, respectively, when compared to CTRL. Moreover, in samples of Adenocarcinoma we documented a 3.8-fold increase in PDGFR$^\text{pos}$ cell density in neoplastic with respect to distal portions of the lung (Figure 5C).

The quantitative estimation of CD34$^\text{pos}$ cells did not show significant difference among the analyzed samples although this parameter tended to increase in neoplastic tissue (Figure 6).

2. In Vitro Study

2.1 IMMUNOCYTOCHEMISTRY

Hematic and lymphatic endothelial cell populations obtained from distal and neoplastic lung were also analyzed in vitro by immunocytochemistry. The expression of CD31 antigen was documented in almost 95% of Blood Endothelial Cell (BEC) population, showing a similar distribution in T and Dist lung portion (Figure 7E).

Lymphatic Endothelial Cells (LEC) were identified by the expression of Podoplanin, LYVE-1 and Prox-1 in CD31 positive cells (Figure 7F).

The expression of tyrosine kinase receptors VEGFR3 and PDGFR$\beta$ was also investigated in LEC population. From 90% to 97% of LEC were labeled by VEGFR3 and PDGFR$\beta$ and their incidence was comparable in cells obtained from neoplastic and distal portions of the lung (Figure 8B-D).

Similar results were obtained for the detection of VEGFR2 and PDGFR$\beta$ in BEC isolated from both samples of the lung (Figure 8A-C).

Importantly, we documented that the nuclear expression of Ki67 was 1.5-fold higher in LEC isolated form neoplastic tissues when compared to those obtained from the spared lung, indicating higher proliferative capacity of vascular cells composing the tumor.
2.2 ANGIOGENESIS ASSAY

A Matrigel®-based tube formation assay was performed to assess the *in vitro* angiogenic properties of human lung BEC and LEC and to determine potential difference between cells isolated from healthy and neoplastic tissues (*Figure 9A-B*). The tubular structures were measured 2, 5, and 24 hours after plating. The quantitative analysis showed that the tube forming ability reaches its peak 24 hours after cell seeding for both cell types. However, different results were obtained from the measurements of BEC and LEC.

In particular, at 24 hours a significant decrease in tube formation was documented in BEC isolated from neoplastic tissues when compared to those obtained from spared lungs (*Figure 9C*). On the other hand, LEC isolated from tumor samples showed a significantly increased in vitro angiogenic ability at early time points (5 hours) but not at 24 hours, when compared to their distal counterpart (*Figure 9D*).

2.3 CELL MIGRATION ASSAY

In order to determine whether endothelial cells isolated from distal or neoplastic lung differ in terms of cell migration, scratch assays were performed. We observed that LEC isolated from Dist and T samples had a similar migratory ability since after 24h the percentage of open wound area was included in a range of 2% to 5% for both cell types (*Figure 10*). At early time point, LEC from Dist seemed to possess increased migratory ability when compared with LEC obtained for neoplastic tissues. Interestingly, the absence of VEGF-C in culture medium did not affect cell migration.
Figure 1: Bar graph showing the volume fraction of total fibrosis measured in distal (Dist) and Neoplastic (T) portion of pathologic and control (CTRL) lungs. * indicates statistical difference vs CTRL, p<0.05.
Figure 2: Microscopic images illustrate immunoperoxidase staining of the endothelial associated CD34 antigen in sections corresponding to the Distal (Dist, A) and neoplastic (T, B) areas sampled from human lungs. Capillary and venule profiles are recognized by the brownish staining of CD34. Nuclei are counterstained by hematoxilin (Scale bars: 100 µm).

C-D: Bar graphs documenting the quantitative measurements of capillaries and venules.

* indicates statistical difference vs CTRL, p<0.05
Figure 3: Microphotographs illustrating arteriolar profiles by the red fluorescence of α-smooth muscle actin in sections of lung Adenocarcinoma. Arteriolar structures are mainly localized in the stroma surrounding neoplastic nodules. Nuclei are recognized by the blue fluorescence of DAPI. Scale Bars: A: 200μm, B: 50μm.
**Arteriolar Density**

**Figure 3C:** Bar graphs documenting the numerical incidence of a-SMA^{pos} arterioles in sections obtained from the distal (Dist) and neoplastic (T) portions of the three lung cancer histotypes. *p<0.05 vs CTRL ;** p<0.05 vs Distal portion.
Figure 4: A: Sections of the alveolar parenchyma of a healthy lung illustrating lymphatic profiles by the green fluorescence of Podoplanin antibody staining. Nuclei are shown by the blue fluorescence of DAPI. Scale Bar: 100 µm.

B: Bar graph showing the quantification of the density of lymphatic vessels in healthy (CTRL), distal (Dist) and neoplastic (T) samples of the human lung.

* indicates statistical difference vs CTRL; ** vs Dist portion, p<0.05.
Figure 5: Microphotographs documenting the immunohistochemical detection of Platelet Derived Growth Factor Receptor (green fluorescence) in lung cancer. Cancer cells are labeled by the red fluorescence of Pan-cytokeratin. Nuclei are shown by the blue fluorescence of DAPI. Scale Bars: A: 100\( \mu \)m, B: 50\( \mu \)m.
Vascular Progenitor Cell Density

Figure 5C: Graphs illustrating the quantitative estimation of the incidence of PDGFR$^{\text{pos}}$ cells in Adenocarcinoma, Squamous Cell Carcinoma and Neuroendocrine Tumor.

* vs CTRL ; ** vs Distal portion, p<0.05
Figure 6: Bar graphs documenting the quantification of CD34$^{\text{pos}}$ individual cells in healthy and pathologic lung tissues.
**Figura 7:** Phase contrast microphotographs showing the similar morphology of Hematic (BEC) and Lymphatic (LEC) Endothelial Cells isolated from Distal (A: BEC, B: LEC) and Neoplastic (C: T-BEC, D: T-LEC) human Lung.

Immunocytochemical detection of CD31 in a typical culture of T-BEC (E, green fluorescence) and of Podoplanin (green) and Prox-1 (magenta) in T-LEC (F). Nuclei are recognized by the blue fluorescence of DAPI. Scale bars: A, B, C, D: 500µm, E: 50µm, F: 100µm.
Figura 8: Microphotographs showing VEGFR-2 (A) and VEGFR-3 (B) expression in BEC and LEC, respectively, isolated from the distal portion of lung cancer. Examples of PDGFRb immunocytochemical detection in T-BEC (C) and T-LEC (D) cultures obtained from Adenocarcinoma. Nuclei are shown by the blue fluorescence of DAPI. Scale bars: A: 50µm; B, C, D: 100µm
Figura 9: Representative images of tube formation on Matrigel by lymphatic endothelial cells isolated from distal (A) and neoplastic (B) lung. Scale bars: A, B: 200 µm
Bar graphs showing the quantitative estimation of the tube forming ability of blood (C, BEC) and lymphatic (D, LEC) endothelial cells isolated from distal and neoplastic (T-BEC, T-LEC) lung tissues.
§ indicates statistical difference vs BEC and LEC, p<0.05
Figura 10: Representative phase-contrast images of cells migrating into the wounded area in an *in vitro* scratch wound healing assay. Wound at time 0 in cultures of LEC form distal (A) and neoplastic (B) lung. Corresponding images taken after twenty-four hours documenting wound repair by both control (C ) and neoeplastic (D) LEC. Scale bars: A, B, C, D: 500µm.
CONCLUSIONS
The specific aim of our study was to establish whether qualitative and quantitative changes in vascular compartments occur in lung cancer.

Our study supports the hypothesis of the presence of vascular rearrangements in lung cancer.

We compared three tumor histotypes (Adenocarcinoma, Squamous Cell Carcinoma and Neuroendocrine Tumor) and in all of them we could document an increased incidence of vascular structures (capillaries and arterioles) when compared to the distal portion and to control samples.

Moreover, vascular progenitor cell distribution was significantly altered in pathologic samples.

In particular, our data showed that in distal portions there was a significant depletion of PDGFR\textsuperscript{pos} cells. On the other hand their incidence was markedly increased within the tumor area. These observations suggest that the tumor or its microenvironment may exert a chemoattractive action on vascular progenitor cells.

The purpose of our in vitro studies on Hematic and Lymphatic Endothelial Cells was not only to characterize their phenotypes in terms of expression of endothelial markers but also to evaluate the presence of Tyrosine Kinase Receptors (i.e. VEGFR2, VEGFR3, PDGFR) which are specific targets of anticancer therapies.

Different implications can be prospectively envisioned by the present investigation. First, the structural alteration and changes in distribution of vascular structures may potentially represent a prognostic index in lung cancer since blood vessel invasion plays a very important role in tumor progression and metastasis. Moreover, our ability to isolate endothelial cells from human neoplastic and normal lung allows studies aimed at the development of specific targeted therapeutic strategies designed at interfering with tumor microenvironment and specifically with cancer stem cell niche.
References


References


