PROGETTO DI RICERCA / RESEARCH PROJECT

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Titolo del progetto / Project title	Tumour microenvironment in Non-Small-Cell Lung Cancer (NSCLC): a proteomic approach to gain insights on novel disease biomarkers and therapeutic approaches.
Corso di dottorato / PhD	ONCOLOGIA E CHIRURGIA SPERIMENTALI

1 - Sommario / Abstract

Lung cancer is one of the leading cause of cancer-related deaths, 85% of which classified as Non-Small-Cell Lung Cancer (NSCLC). Until few years ago advanced chemotherapy was the only available solution, characterized by limited efficacy [1]. Recently, treatments have been improved with the introduction of “immunotherapy”, consisting in new drugs directed towards specific molecular targets (targeted therapy). Immune checkpoint inhibitors (ICIs) have dramatically changed the landscape of NSCLC treatments. Furthermore, the results of new clinical trials contributed in understanding the mechanism of action of these novel agents, and which class of patients are more likely to benefit. Consequently, ICIs are now part of the first-line NSCLC treatment armamentarium both as monotherapy or combined with chemotherapy.

The most representative ICIs include blockades for programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) [2]. The expression of PD-L1 in malignant cells as a potential biomarker for response to ICIs has been investigated, though a better characterization of its action is necessary. The accurate identification of predictive biomarkers beyond programmed cell death protein-ligand 1 expression is essential to select the most appropriate candidates for ICI therapy [3]. Also, identification of predictive and prognostic, non-invasive markers is necessary. Recent preclinical studies have shown that is possible to isolate exosomes and collect other soluble molecules from human fluids in some tumour types [4], [5].

The goal of this study is to identify new predictive and/or prognostic NSCLC biomarkers through a bottom-up proteomic approach, and to shed new light on protein protagonists of the complex tumour microenvironment in NSCLC patients. The study might contribute to improve patient stratification through the identification of specific molecular signatures enabling personalized therapies.

2 - Descrizione del progetto / Project

Project Outline: State of Art Lung cancer is one of the most common cancers and the main cause of death from cancer. It is a subtle disease, where the prognosis is often poor. There are two main types of lung cancer: small-cell or microcytoma (SCLC) and Non-Small-Cell Lung Cancer (NSCLC) with an incidence rate of 10% and 85%, respectively. Only 5%

of lung cancer cases are instead classified as pulmonary carcinoid of neuroendocrine origin, or as pulmonary lymphoma (of lymphatic origin) or as pleural mesothelioma.

Furthermore, NSCLC shows cellular heterogeneity, with three tumour cell subtypes: Squamous cell carcinoma (or squamous); Adenocarcinoma; Large cell carcinoma.

These three cancer subtypes arise from various genetic alterations that occur with different frequencies. For example, in patients diagnosed with NSCLC, 15% is mutated in EGFR, 15% in K-RAS and 5% in ALK. This indicates a heterogeneity on the population affected by NSCLC. Furthermore, according to the American Cancer Society there is a heterogeneity even within each specific mutation [6].

The treatment choice strongly depends from the cancer stage, and molecular signatures of each patient can predict clinical benefits. A finest patient classification for the different treatment options could avoid unnecessary toxicities and costs, thus improving the benefits [7]. The use of novel drugs with innovative mechanisms of action, such as antiangiogenic compounds and molecular therapies, results in a more effective treatment for this aggressive disease. Several clinical trials showed that reversible EGFR tyrosine-kinase inhibitors (TKIs), Erlotinib and Gefitinib, give better response rates and longer progression-free survival vs standard chemotherapy regimens in first-line treatment [8,9,10]. In addition, patients with ALK translocation respond to ALK tyrosine kinase inhibitors, such as Crizotinib [11]. However, EGFR TKI and ALK inhibitors are not curative because resistance against these drugs arises [12,13].

Recently, a therapeutic revolution consisting in molecular targeted drugs and immunotherapy has gained a crucial role in the treatment of these patients. Indeed, neutralizing antibodies targeting immune checkpoint programmed cell death protein 1 (PD-1) or its ligand (PD-L1) have been particularly successful for tumour types with limited therapeutic options [2].

These inhibitors, alone or in combination, improve the response and the progression-free survival (PFS) for NSCLC patients, compared with chemotherapy alone. Indeed, improving patients classification will bring better clinical outcomes from immunotherapy, though the characterization of patients is still not definite. Till today, PD-L1 expression on tumour tissue has been the only officially approved biomarker for patient selection, but the substantial heterogeneity of spatial and temporal PD-L1 expression pattern suggests that PD-L1 alone might not be sufficient for patient selection [14]. Thus, novel biomarkers are essential for immunotherapy indication in patients. Biomarkers recognized as potential NSCLC candidate include PD-L1, cancer-driven mutations, Tumour-Infiltrating Lymphocytes (TILs) and Tumour Mutational Burden (TMB), defined as the total number of somatic mutations per megabase. Unfortunately, some studies have shown the clinical significance of TMB in predicting immunotherapy efficacy, while some other yielded contradictory results [15,16,17].

A more effective selection for cancer biomarkers is needed for new therapeutic approaches, and is dedicated to the study of cancer cell secretome as a tool to identify diagnostic and prognostic markers and potential therapeutic targets [18].

The “secretome” is referred to as the rich, complex set of molecules secreted from living cells including molecules shed from the surface of living cells and extracellular vesicles (exosomes) playing a key role in cell signaling, communication and migration [19]. Exosomes are small (40 to 100 nm) extracellular vesicles released from normal, diseased, and neoplastic cells and are present in the blood and other body fluids. Exosome contents include a variety of molecules, such as proteins, signal peptides, mRNA, microRNA and lipids. The effects of exosomes on the development and progression of cancer and metastasis is well described, as well as their potential use in the development of vaccines, treatment, diagnosis and follow-up [20].

There are several ongoing studies focusing on genetic and transcriptomic factors as biomarkers for cancer disease, while only few studies based on protein biomarkers are available. Being proteins essential mediators in biology and representative for healthy and diseased phenotype, they should be taken into account.
Emerging sensitive proteomic based technologies, such as liquid chromatography mass spectrometry (LC-MS)-based quantitative proteomics can provide a platform for evaluating serial serum or plasma samples to interrogate secreted products of tumour–host interactions, thereby revealing a more "complete" repertoire of biological variables encompassing heterogeneous tumour biology [21].
Referred as Bottom-up proteomics, this technique utilizes the advantages that peptides have over proteins: peptides are more easily separated by reversed-phase liquid chromatography using a HPLC, ionize well, and fragment in a more predictable manner. This translates into a robust methodology that enables high-throughput analysis, allowing for identification and quantification of thousands of proteins from complex samples. Also termed shot-gun proteomics, these straightforward workflows generate large lists of protein identifications and were used for solving most of the available, complex, full proteomes available today, including the first drafts of the human proteome [22,23].

Methodology: The in vitro study of this project will first make use of different cell lines (see table 1), representing the subtypes of lung cancer. These cell lines will be cultured according to the appropriate conditions for each cell line. Next, the secretome of these cells will be isolated, both will be examined with proteomic techniques. Proteins secreted in secretome and proteins derived from the exosome fractions will be extracted and digested into tryptic peptides. The resulting peptides will be separated on a nano-HPLC and analyzed online on the Thermo Q-exactive mass spectrometer.

Cell Line	Lung cancer subtype	Mutation
LUDLU-1	Squamous	-
ECB-1	Squamous	MET amplification
A549	Adenocarcinoma	-
A549	Adenocarcinoma	K-Ras G12S mutation
CRL-5883	Adenocarcinoma	EGFR (DelE746A750)
CRL-5878	Large cell	-
H1975	Adenocarcinoma	T790M in-cis with L858R EGFR mutation
H460	Large cell	K-Ras G12S mutation
HCC827-GR5	Adenocarcinoma	EGFR (DelE722-726) and Met amplification

Table 1: Cell lines used in the project

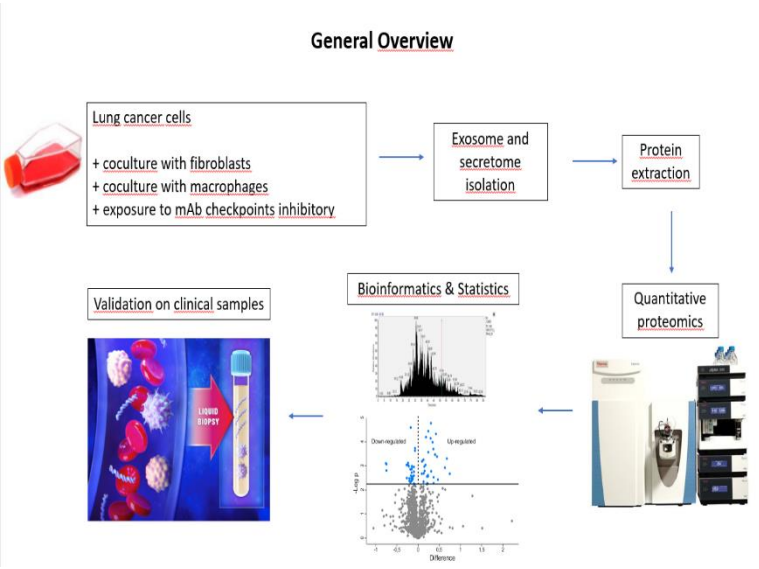
For identification and quantification of the occurring peptides in secretome and exosome samples, a label free approach will be used, which by querying a database, will give us a shortlist of differential proteins and peptides will be generated to be validated as potential biomarkers in clinical samples.
Once a list of potential biomarkers (proteins, peptides) is generated, they can be validated in clinical samples. Recent preclinical studies have identified that it is possible to isolate exosomes and secretomes from human fluids in some types of cancer, including NSCLC, through liquid biopsies: an innovative, non-invasive and performing approach to analyze the tumor structure from a molecular point of view. We can define liquid biopsy as the use of different biological fluids as surrogates for a neoplastic tissue.

WP1: Lung cancer cell Lines
The goal is to analyze exosomes and secretomes of *in vitro* cultured stable lung cancer cells. Selected cell lines will be cultured according to the appropriate conditions. Then, cell secretome will be collected, exosomes will be isolated from secretome (e.g, by differential ultracentrifugation), and both secretome and exosomes will be examined with proteomic approaches. In addition, a genetic analysis will validate the results.
WP2: Cocultures of lung cancer cell lines with fibroblasts or macrophages

This part is designed to analyse the effect of drug treatment on the secretome and exosome content, with the same proteomic techniques as described above. Cocultures of lung cancer cell lines with fibroblast or tumor-associated macrophages will be established using a transwell apparatus in order to assess the influence of cells and the microenvironment on tumor cell secretome and exosome contents. Moreover, different cell lines will be exposed to drugs/mAb immunecheckpoint inhibitors (e.g. Nivolumab, Pembrolizumab to target PD-1; or Atezolizumab and Durvalumab to hit PDL-1).

WP3: Clinical data validation

The final research hypothesis of this project is to establish the potential value of secretome and exosome proteomics as predictive and prognostic markers by an easy non-invasive method in NSCLC patients in early and metastatic setting. For this purpose, liquid biopsy from human samples will be analyzed to validate the discovered biomarkers through the development of robust and accurate computational tools.



Feasibility

The proposed project will be carried out as part of the Ri.MED Foundation at the IRCCS ISMETT (Mediterranean Institute for Transplantation and Advanced Specialized Therapies). Ri.MED Foundation, IRCCS ISMETT and their partner University of Pittsburgh Medical Center (UPMC) form a cluster of excellence promoting translational research, which combines high-profile scientists with expertise in cancer and immunotherapy, oncologists and cancer care, onco-surgeons and a Good Manufacturing Practice (GMP) facility. The team includes a specialized technical staff for designing and performing preclinical/clinical trials and producing advanced therapy medicinal products (ATMPs) for cancer immunotherapy.

In this scenario, Ri.MED has a proteomic platform led by Dr. Simone Scilabra, which will be dedicated to the proteomic analysis of secretome and exosomes based on a nano-LC-MS/MS setup that includes an Ultimate 3000 HPLC system and a Q-Exactive mass spectrometer.

As part of the Proteomics Unit at Ri.MED, I will be able to perform protocols, such as Integrated in-solution digestion methods including filter-aided sample preparation (FASP) and an StageTip desalting treatment [24,25,26].

3 - Bibliografia / References

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